INVESTIGATIONS ON RAB31'S ROLE IN EGFR TRAFFICKING AND NEURAL PROGENITOR CELL DIFFERENTIATION TOWARDS ASTROGLIA

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B. Sc (Hons.), NUS

A THESIS SUBMITTED FOR

THE DEGREE OF DOCTOR OF PHILOSOPHY

NUS GRADUATE SCHOOL FOR INTEGRATIVE SCIENCES AND ENGINEERING

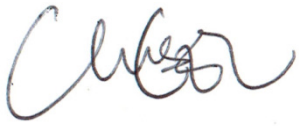
NATIONAL UNIVERSITY OF SINGAPORE

2014

Declaration

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in the university previously.



Christelle Chua En Lin

11 April 2014

Acknowledgements

I would like to thank my supervisor, A/Prof Tang Bor Luen, for his unwavering support and mentorship through the course of my studies.

I would also like to acknowledge our past and present lab members, for their expertise and assistance which have contributed to my research.

Lastly, I would like to extend my gratitude to my family and friends for their support and encouragement.

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Summary

Rab31 is a member of the Rab5 subfamily of Rab GTPases, which play a role in trafficking of endocytic lumenal and membrane cargo. We have investigated factors influencing Rab31's subcellular localisation and found that Rab31 functional domains and interacting partners are both important to its localisation at the trans-Golgi network (TGN). We also investigated Rab31's role in the trafficking of ligand-bound epidermal growth factor (EGF) receptor (EGFR) internalised through receptor-mediated endocytosis, which has hitherto not been explored. We found that depletion of Rab31 inhibits, while Rab31 overexpression enhances, EGFR trafficking to the late endosomes. Rab31 was found to interact with EGFR by co-immunoprecipitation and affinity pulldown analyses, and the primarily TGN-localised Rab31 has increased colocalisation with EGFR on endosomes at 30 min after pulsing with EGF. We found that loss of early endosome antigen 1 (EEA1), a Rab31 effector, reduced the interaction between Rab31 and EGFR, and abrogated the effect of Rab31 overexpression on the trafficking of EGFR. Likewise, loss of GAPex5, a Rab31 guanine nucleotide exchange factor (GEF) that has a role in ubiquitination and degradation of EGFR, reduced the interaction of Rab31 with EGFR and its effect on EGFR trafficking. Taken together, our results suggest that Rab31 is an important regulator of endocytic trafficking of EGFR, and functions in an EGFR trafficking complex that requires EEA1 and GAPex5 for its formation. To explore the physiological role of Rab31 which is highly expressed in radial glia and mature astrocytes, we looked at Rab31 in neural progenitor cells (NPCs) both in the neurogenic regions of the adult mouse brain and in culture. NPCs expressed high levels of Rab31, but when NPCs were induced to differentiate, Rab31 levels dipped then reappeared in a subset of the glial fibrillary acidic protein (GFAP)-positive

astrocyte population. Depletion of Rab31 appeared to decrease the percentage of GFAP-positive cells obtained. This suggests that Rab31 plays a role in the differentiation of neural progenitor cells of the brain. This may be, in part, due to its role in EGFR trafficking. Results presented in this thesis have implications for both our understanding of neurogenesis and cancer therapeutics.

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List of Symbols

kb - kilobase

kDa - kilodalton

μg - microgram

μm - micrometer

mL - millilitre

mM - millimolar

ng - nanogram

nM - nanomolar

μg/mL - microgram per millilitre

mg/mL - milligram per millilitre

ng/mL - nanogram per millilitre

^{o}C - degree Celsius

List of Abbreviations

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AP	adaptor protein
APPL	adaptor protein, phosphotyrosine binding domain, pleckstrin homology domain, leucine zipper containing proteins
ATCC	American Type Culture Collection
BSA	bovine serum albumin
Cbl	Casitas B lineage lymphoma
cDNA	complementary DNA
CIP4	Cdc42-interacting protein
CNPase	2', 3'-cyclic nucleotide 3'-phosphodiesterase
CNS	central nervous system
co-IP	co-immunoprecipitation
COP	coat protein
DCX	doublecortin
DENN	differentially expressed in normal and neoplastic cells
DG	dentate gyrus
DMEM/F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DNA	deoxyribonucleic acid
E15	embryonic day 15
EE	early endosome
EEA1	early endosome antigen 1
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein

EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
ERα	estrogen receptor α
EV	endocytic vesicle
FBS	fetal bovine serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
GA	Golgi apparatus
GAP	GTPase activating protein
GDF	GDI displacement factor
GDI	GDP dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFAP	glial fibrillary acidic protein
GG	geranyl-geranyl
Grb	growth factor receptor-bound protein
GST	glutathione S-transferase
GTP	guanosine triphosphate
HEPES	hydroxylethyl piperazineethanesulfonic acid
HEK	human embryonic kidney
HOPS	Hsp70-Hsp90 Organising protein system
HV	hypervariable domain
HRP	horseradish peroxidase
IACUC	Institutional Animal Care and Use Committee
IMAGE	Integrated Molecular Analysis of Genomes Consortium

LDL	low density lipoprotein
LE	late endosome
L/V	lysosome / vacuole
M6PR	mannose 6-phosphate receptor
MAPK	mitogen activated protein kinase
MMP	matrix metalloproteinase
mRNA	messenger RNA
MUC1	mucin-1
NPCs	neural progenitor cells
NSF	n-ethylmaleimide sensitive factor
NUS	National University of Singapore
OCT	Optimum Cutting Temperature
OE	overexpressing
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PDL	poly-D-lysine
PI3K	phosphotidylinositol-3-kinase
PI3P	phosphotidyl inositol 3-phosphate
PLC	phospholipase C
PM	plasma membrane
PMSF	phenylmethylsulphonyl fluoride
Rab	Ras-related protein in brain
RE	recycling endosome

REP	Rab escort protein
RGGTase	Rab geranylgeranyl transferase
RILP	Rab-interacting lysosomal protein
RIN	Ras and Rab interacting protein
RNA	ribonucleic acid
RT	reverse transcription
Scr	scrambled
SEM	standard error of the mean
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH	Src homology
shRNA	small hairpin ribonucleic acid
siRNA	small interfering ribonucleic acid
SNARE	soluble NSF attachment protein receptor
SV	secretory vesicle
TBC	Tre2/Bub2/Cdc16
TCA	trichloroacetic acid
TGF	transforming growth factor
TGN	trans-Golgi network
TIP47	tail interacting protein of 47kDa
TRAPP	transport protein particle complex
TxR	Texas Red
VPS	vacuolar protein sorting

1. Introduction

1.1 Overview of Rab GTPases

GTPases are GTP-activated regulatory proteins with an intrinsic enzymatic activity that hydrolyses guanosine triphosphate (GTP) to guanosine diphosphate (GDP). The superfamily of small (20-35 kDa) GTPases include Ras, Rho, and Rab, and these function as molecular switches in the cell, with the latter playing critical roles in membrane transport (Colicelli, 2004). Rabs (Ras-related protein in brain) are found in all eukaryotes, including yeast, plants and mammals (Pfeffer, 2005) and the human genome encodes over 60 different Rab and Rab-like genes (Segev, 2001; Hutagalung and Novick, 2011; Klöpper et al., 2012).

Rab proteins are peripheral membrane proteins. They have a hydrophobic prenyl (geranyl-geranyl) group attached to two terminal cysteines at the C-terminus. When the Rab GDP dissociation inhibitor (GDI) is bound to the prenyl group, the GDP-bound Rab remains in the cytosol and is inactive (Fig. 1.1A). Removal of GDI exposes the prenyl group, allowing the Rab to be inserted into the target membrane (Fig. 1.1B). A Rab guanine nucleotide exchange factor (GEF) exchanges GDP for GTP, resulting in an active, membrane bound Rab which, in its active conformation, can then recruit its effector proteins (Fig. 1.1C). A GTPase-activating protein (GAP) activates the intrinsic GTPase activity of the Rab, which hydrolyses GTP to GDP, enabling the Rab to be extracted from the membrane by GDI (Fig. 1.1D) (Nottingham and Pfeffer, 2009; Schwartz et al., 2008; Chua and Tang, 2011; Ng and Tang, 2008).

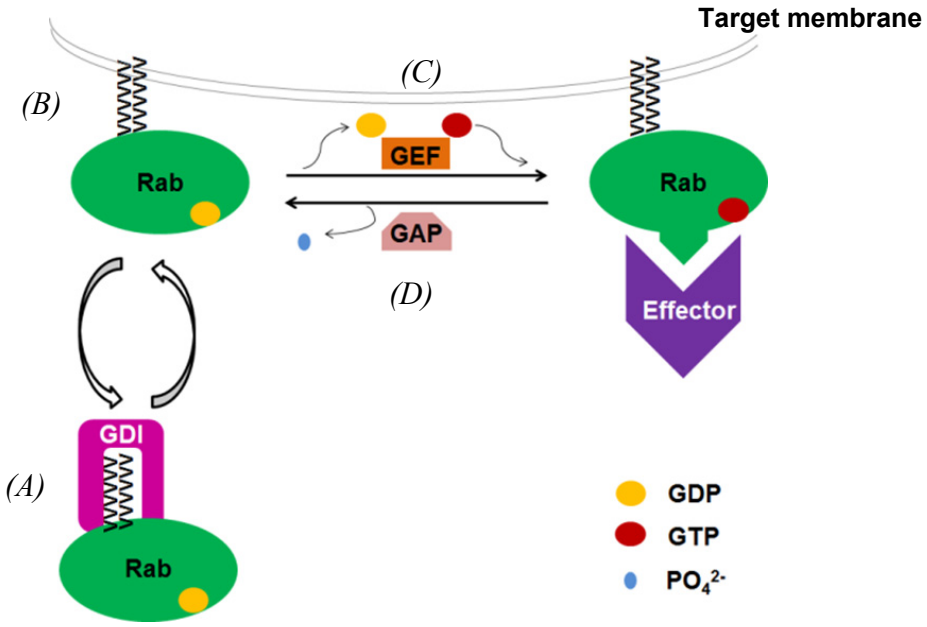


Fig. 1.1. The Rab guanine nucleotide cycle
Rab proteins cycle between their GDP and GTP bound state, and move between the cytosol and their target membrane. Refer to text for description. GEF: guanosine nucleotide exchange factor; GAP: GTPase activating protein; GDI: GDP dissociation inhibitor; GDP: guanosine diphosphate; GTP: guanosine triphosphate; PO_4^{2-}: phosphate ion.

The eukaryotic cell is highly compartmentalised, and specific transport processes occur between the different compartments (Deneka et al., 2003). In the exocytic pathway, anterograde transport of endoplasmic reticulum (ER) targeted nascent protein occurs as they are transported from the ER to the Golgi apparatus (GA). Extracellular or membrane proteins exit the GA and are then sorted at the trans-Golgi network (TGN) into secretory vesicles (SV), while intracellular proteins are sorted to their various organelles. In the endocytic pathway, retrograde transport occurs. Endocytosis is the process by which the plasma membrane of a cell invaginates, enabling it to internalise extracellular fluids, proteins and other signalling molecules into vesicles which fuse to form early endosomes. Sorting at the endosomal network takes place, and proteins such as signalling molecules may be targeted for degradation through late endosomes (LE) / lysosomes, while receptors may be

recycled back to the cell surface via recycling endosomes (RE). Traffic also occurs between the Golgi and the various membranous compartments such as the ER and the endosomal network, and is a means by which proteins with functions in the compartments themselves are targeted and recycled (Fig. 1.2).

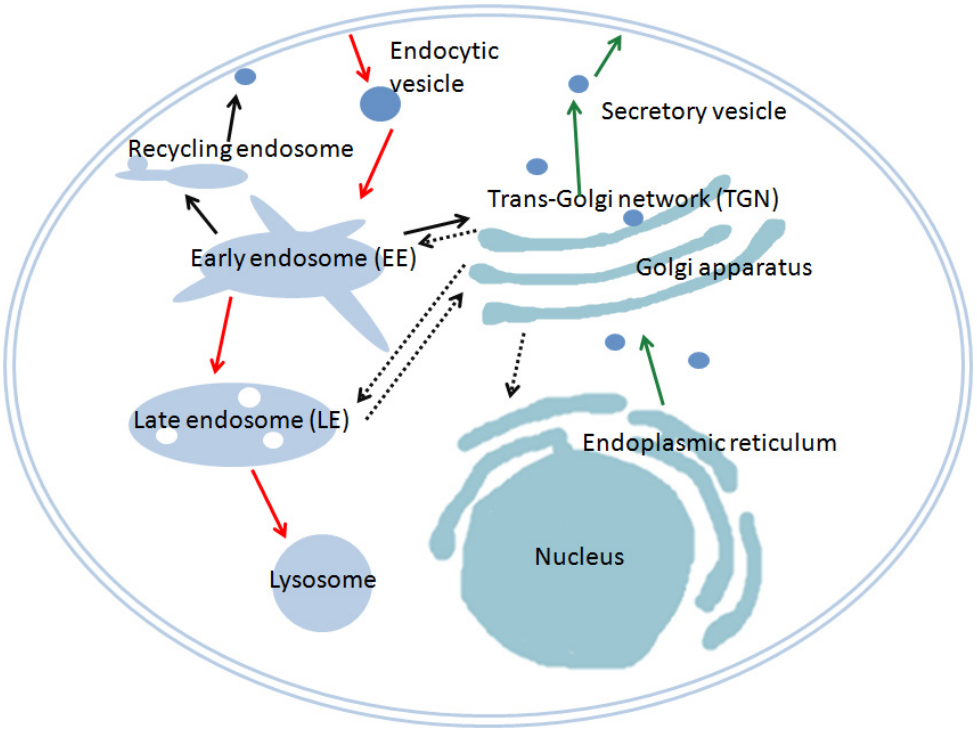


Fig. 1.2. Schematic diagram of intracellular membrane trafficking pathways
The endocytic pathway involves retrograde transport (red arrows) and the exocytic/biosynthetic pathway involves anterograde transport (green arrows). Cargo at the early endosome can also be recycled to the plasma membrane or returned to the TGN (black arrows). Proteins localised to various membranous compartments are themselves trafficked and recycled to and from the Golgi (dotted arrows). Refer to text for more details. ER: Endoplasmic reticulum; GA: Golgi apparatus; TGN: trans-Golgi network; SV: Secretory vesicle; EV: Endocytic vesicle; EE: Early endosome; RE: recycling endosome; LE: late endosome; L/V lysosome/vacuole.

Rabs confer specificity to particular vesicular transport steps by virtue of their large repertoire of specific interacting proteins as well as via their specific subcellular location along the vesicular transport pathways (Segev, 2011; Grosshans et al., 2006). The following sections discuss how Rabs regulate membrane traffic in more detail.

1.1.1 Rab-interacting proteins and how they aid in achieving specificity in Rab function

As discussed above, Rabs interact with a variety of regulatory proteins which serve to activate or deactivate them, as well as effector proteins that act downstream. Specificity of Rab function is thus conferred by these proteins, which are interacting partners to specific Rabs or subfamilies of Rabs.

a) Rab guanine nucleotide exchange factors (GEFs)

GDP dissociates from Rab proteins at a very low rate, and GEFs serve to catalyse the exchange of GDP for GTP on the Rab protein by altering the conformation of the nucleotide binding site, promoting GDP dissociation. This then enables GTP, which exists in much higher concentrations in the cell, to associate with the Rab. GEFs thus aid in the activation of the Rab. In general, they have a higher affinity for the GDP-bound form of the Rab, and therefore dissociate from the Rab once it is GTP-bound and activated. One Rab can have many activating GEFs. For example, Rab5's GDP-GTP exchange could be aided by GAPex5 (Hunker et al., 2006) (also known as RME-6 in *C. elegans* (Sato et al., 2005)), Ras and Rab interactor 1 (RIN1) (Tall et al., 2001), Rabex5 (Horiuchi et al., 1997), and Alsin (Topp et al., 2004).

That these GEFs serve different cellular functions is hinted at by the fact that they have other functional and/or regulatory domains other than the GEF domain, and also exhibit different subcellular localisation within the cell (van der Bliek, 2005). To elaborate further, various protein domains have been delineated to be associated with Rab GEF function. Amongst them is the Vps9 domain, which serves as a GEF for members of the Rab5 subfamily, and is found in 18 mammalian proteins (Delprato et al., 2004; Carney et al., 2006). One example is the Ras and Rab interactor (RIN) family

of proteins, which besides the Vps9 domain also contains a Ras association (RA) domain that enables Ras-dependent allosteric regulation of the proteins' GEF activity (Tall et al., 2001; Bliss et al., 2006; Yoshikawa et al., 2008). This suggests that regulatory mechanisms of GEFs exert spatial and temporal control of Rab activity.

The Differentially Expressed in Normal and Neoplastic cells (DENN) domain is another putative GEF domain. The DENN domain of the connecdenn family of proteins acts as a GEF for Rab35 (Allaire et al., 2010). Several different functions have been attributed to Rab35, including fast recycling on early endosomes, and modulation of actin dynamics via the actin bundling protein fascin, a Rab35 effector (Marat et al., 2012; Chua and Tang, 2011). It is proposed that different DENN domain proteins act as GEFs for Rab35 in specific contexts to control the diverse functions of the Rab (Marat and McPherson, 2010). Again, this suggests that the different GEFs also aid in determining the specificity of Rab function.

b) Rab GTPase activating proteins (GAPs)

GAPs terminate the activity of Rab proteins by stimulating the intrinsically low Rab GTPase activity to hydrolyse bound GTP. GAPs bind to Rabs and induce a conformational change in the Rab that exposes the GTP to facilitate nucleophilic attack by water, which hydrolyses the GTP by breaking the phosphate bond. To date, all identified Rab GAPs contain a Tre2/Bub2/Cdc16 (TBC) domain (Pan et al., 2006; Fukuda, 2011). Over 50 TBC domain-containing proteins have been found in humans, but their functions are poorly characterised (Gabernet-Castello et al., 2013). It is believed that a conserved arginine finger within the domain interfaces with the Rab nucleotide binding pocket, which stimulates GTP hydrolysis (Hutagalung and Novick,

2011). GAPs also aid in the specificity and function of Rabs by determining their subcellular localisation, discussed later below.

c) Rab Effectors

Effector proteins refer generally to any protein that interacts with the activated, GTP-bound Rab. Rabs regulate vesicular transport in the cell by engaging various effector proteins, such as motor proteins, tethering factors, and fusion components such as SNAREs (soluble N-ethylmaleimide sensitive factor (NSF) attachment protein (SNAP) receptor) (Markgraf et al., 2007; Seabra and Coudrier, 2004; Li, 2001; Simonsen, 1999).

Rabs aid membrane vesicle motility by recruiting motor proteins. Rab7 mediates fusion between late endosomes and lysosomes. One of its effectors is the intermediate filament associated protein, Rab-interacting lysosomal protein (RILP), which recruits dynein-dynactin motor complexes to the late endosome and lysosomes and enables the transport towards the minus-end of microtubules (Jordens et al., 2001).

Many Rabs have also been shown to recruit tethering factors, which facilitate the docking and subsequent fusion of vesicles. For example, Rab1 has been shown to bind p115, which aids in the fusion of ER-emerging COPII vesicles with the Golgi membrane (Beard et al., 2005; Grabski et al., 2012). Rab5 has been shown to bind early endosome antigen 1 (EEA1), a tethering factor which mediates homo- and hetero-typic fusion between early endosomes (Dumas et al., 2001; Simonsen, 1999).

Rabs also engage SNAREs, which are responsible for mediating vesicular fusion (Stenmark, 2009; Segev, 2011). Rab5 was found to assemble into a large oligomeric complex which includes the Rab5 interacting proteins EEA1, Rabaptin5, and Rabex5, as well as the SNARE interacting protein N-ethylmaleimide sensitive factor (NSF).

Syntaxin13 interacts with this complex (McBride et al., 1999). Using reconstituted proteoliposomes, it was later confirmed that Rab5 helps to stabilise the presence of the syntaxins on the membranes to aid fusion (Ohya et al., 2009).

Table 1.1 lists some known Rab effectors and how these exert their roles in membrane transport.

Effector Type	E.g.	Rab	Function	Ref.
Motor protein	Microtubule motor Rabkinesin 6	Rab6	GA to ER transport	(Echard, 1998)
Intermediate motor protein	Melanophilin	Rab27A	Links myosin Va to melanosome; retains melanosomes at the actin network in cell periphery	(Strom, 2002)
Coiled-coil tethers	P115 and GM130	Rab1	Tether ER vesicles to Golgi	(Allan, 2000; Moyer et al., 2001)
	EEA1	Rab5	Tether EE vesicle for fusion	(Christoforidis et al., 1999)
Large subunit tethers	Octameric Exocyst complex subunit Sec15	Rab11	Tether secretory vesicles to PM	(Zhang et al., 2004)
	Conserved oligomeric Golgi complex (COG)	Rab1	Interacts with COPI coat; aids in retrograde traffic at Golgi	(Suvorova, 2002)
SNAREs	SNAP29	Rab3A	Trafficking of myelin in glia	(Schardt et al., 2009)
	Syntaxin4	Rab4	GLUT4 translocation	(Li, 2001)

Table. 1.1. Rab effector proteins
Examples of types of effector proteins and their Rabs. GA – Golgi apparatus, ER - endoplasmic reticulum, EE – early endosome, PM – plasma membrane. See text for more details.

1.1.2 Localisation of Rab proteins to target membranes

Another aspect of Rab specificity lies in their specific subcellular localisation within the cell. Rabs function while associated with their respective target membranes (Fig. 1.3). How Rabs are targeted specifically to a particular membrane is therefore of great interest. Conceivably, this is partly dependent on the Rab-interacting proteins, but also on structural domains within the Rab proteins. Newly synthesised Rabs are soluble cytosolic proteins that are post-translationally modified by prenylation (typically the addition of two geranyl groups) to its C-terminal cysteine residues linked by thioether bonds. This is mediated by Rab escort proteins (REP), which chaperone the newly synthesised Rabs to Rab geranylgeranyl transferases (RGGTase) (Pereira-Leal et al., 2001). After prenylation, Rabs can be lipid-anchored into their target membranes, where they undergo GEF-mediated GDP-GTP exchange and engagement of effector proteins. GAPs enhance the intrinsic GTPase activity of the Rabs, resulting in inactivation (Nottingham and Pfeffer, 2009). GDP-bound Rabs can then be extracted from the membrane by GDP dissociation inhibitors (GDI), which have higher affinity for the GDP-bound form of prenylated Rab (Ullrich et al., 1993; Ullrich et al., 1994; Soldati et al., 1994). Through a variety of different speculative mechanisms, Rabs can then be dissociated from GDI and reinserted into the membrane to repeat the Rab cycle (Fig. 1.1).

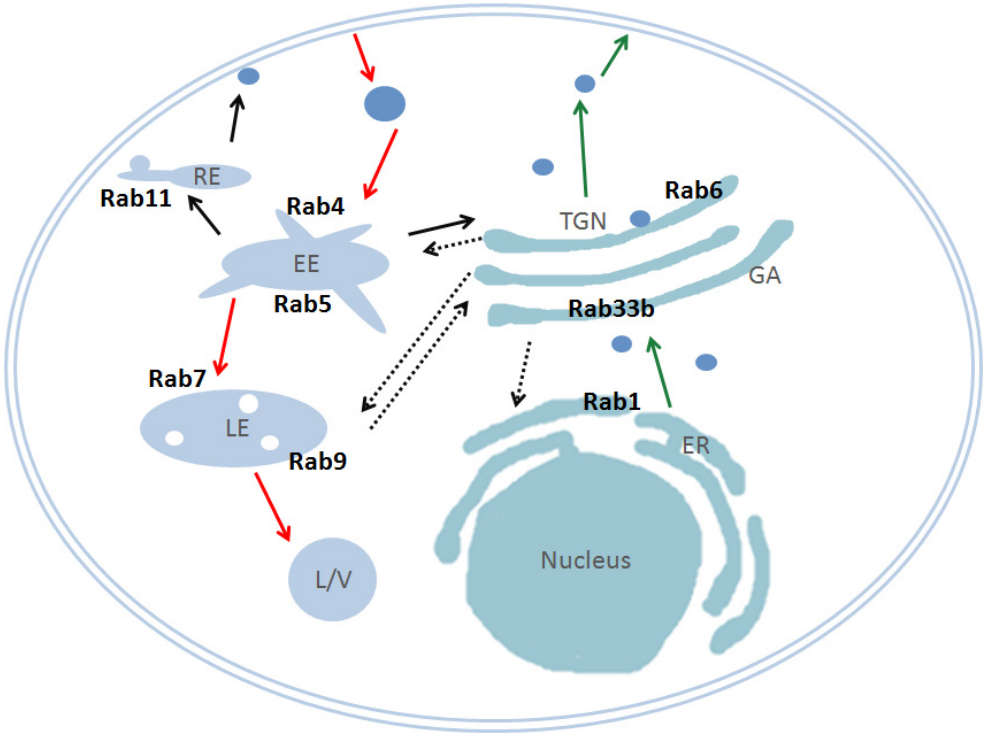


Fig. 1.3. Schematic diagram illustrating subcellular localisation of various Rab proteins
Examples of different Rabs and their unique subcellular localisation. ER: Endoplasmic reticulum; GA: Golgi apparatus; TGN: trans-Golgi network; EE: Early endosome; RE: recycling endosome; LE: late endosome; L/V lysosome/vacuole.

Different Rabs largely occupy different subcellular compartments, and even within the same membrane compartment, different Rabs may occupy different membrane microdomains. For example, Rab5 and Rab4 both exist on endosomes but occupy different microdomains and have different functions. Rab5 typically mediates homo- and heterotypic endosomal fusion while Rab4 mediates fast recycling directly to the plasma membrane (De Renzis et al., 2002). Rab7 and Rab9 also occupy separate microdomains on late endosomes, and also mediate separate processes. Mannose 6-phosphate receptors (M6PR) carry newly synthesised lysosomal enzymes from the TGN to late endosomes/lysosomes, and is itself recycled to the Golgi, a step which is mediated by Rab9 (Barbero et al., 2002). M6PR has been seen to concentrate in Rab9

microdomains. In contrast, Rab7 has been shown to play a role in the degradation of cargo from the plasma membrane that enters the endocytic-degradative pathway (Feng et al., 1995; Vanlandingham and Ceresa, 2009).

Many different mechanisms, based both on Rabs' primary structure and Rab interacting proteins, have been put forth to postulate how Rabs are first targeted to their specific membranes / organelles and then held there in microdomains by interactions with a variety of proteins. The following discusses some of these mechanisms:

a) Rab hypervariable domain

The Rab hypervariable (HV) domain refers to the sequence variability of approximately 35-40 amino acids that exists between the last α-helix and the C-terminal prenylation region. It is especially important for the hydrophobic interactions with GDI (Pfeffer, 2005), which binds at the top to switch regions and at the bottom to the prenylation group (Pfeffer and Aivazian, 2004). Evidence for the importance of the HV domain in determining Rab specificity has been varied. For example, in terms of engaging interacting proteins, the HV domain of Rab5 has been shown to be dispensable for EEA1 interaction (Merithew et al., 2003; Mishra et al., 2010). Moreover, as evidenced by members of the Rab5 subfamily (described in more detail in Section 1.2), Rabs with different HV domains can still have the same effectors and, to some extent, similar subcellular localisations. With regards to whether the HV domain is important to Rab subcellular location, in early studies using Rab5 chimeric proteins consisting of the Rab5 backbone and the Rab7 HV domain, no mistargeting of the Rab5 protein was observed. However, a deletion of the HV domain did render the protein cytosolic despite the presence of the C-terminal cysteines (Chavrier et al., 1991),

suggesting that the HV region may still be important for correct folding, or post-translational modifications before a Rab can become membrane-associated. Hence, while the HV domain may not be necessary for specific membrane targeting *per se*, it is important for membrane association. More recent studies using chimeric proteins have also suggested that the HV domain was dispensable for correct membrane localisation (Ali et al., 2004). However, in one study, it was shown that the Rab9 HV domain was necessary and sufficient for binding to its effector, Tail-interacting protein of 47kDa (TIP47) (as long as a Rab backbone is also present). Chimeras consisting of Rab5 or Rab1 backbone with Rab9 HV domain were all able to interact with TIP47 (Aivazian et al., 2006). As discussed below, TIP47 was subsequently found to be the interacting partner responsible for correct membrane targeting of Rab9. It is pertinent to mention, however, that the converse need not be true – the Rab5 HV domain appeared to play little importance in the interaction with its effector EEA1, nor its early endosome localisation. Thus, it would seem that the HV region varies in importance for specificity of Rab membrane association, depending on the particular Rab protein.

b) Rab prenylation and prenylation machinery:

Dual prenylation of Rabs has also been shown to be important for specific targeting, as Rabs which have been altered to have only a single prenylatable C-terminal cysteine were mistargeted to the ER (Gomes et al., 2003; Calero et al., 2003). The exact reasons for this is unknown, but has been postulated to occur due to the specific requirements for REPs and RGGTs, which may associate with other factors important for correct membrane targeting. It is postulated that REPs may be responsible for the initial targeting of the new Rab to the correct membrane (Ali et al.,

2004), but no direct evidence for this currently exists. Moreover, because only a few REPs have been identified (there are only two isoforms in mammalian cells) (Goody et al., 2005) and are believed to serve a wide variety of Rabs, it would seem unlikely that specificity is conferred solely by the REPs.

c) GDP dissociation inhibitors (GDI) and GDI displacement factors (GDF):

Conserved residues on the GDI as well as those on the switch regions of Rabs enable a few GDIs to recognise an extensive range of Rab proteins. As such, it is unlikely that a few GDIs could be responsible for the wide variety of locations to which Rabs are targeted. However, there is some evidence that the GDI is important to membrane association. Purified Rab5-GDI complexes added to Streptolysin O-permeabilised Madin-Darby canine kidney cells enabled Rab5 to be inserted into membranes, whereas non GDI-bound Rab5 aggregated (Ullrich et al., 1994). Similarly, Rab9-GDI complexes could load Rab9 onto membranes isolated from the late endosome but not the ER, suggesting that the Rab-GDI complexes may carry some information on subcellular membrane specificity (Soldati et al., 1994).

There is also evidence for the existence of a GDI displacement factor (GDF), which would help to dissociate the GDP-bound Rab from the GDI at the target membrane. A protein factor purified from endosomes was shown to catalyse the release of GDP from the GDI-Rab9 complex in reconstituted liposomes, enabling GTP binding. As it did not enhance the intrinsic nucleotide exchange of prenylated Rabs, it was therefore not a GEF, and was instead termed a GDF. Interestingly, it also catalysed the GDP release of other endosomal Rabs such as Rab5 and 7, suggesting perhaps that the localisation of the GDF defines, in part, its specificity of action, and more importantly, may help target endosomal Rabs to their correct location (Dirac-

Svejstrup et al., 1997). Another protein, Yip3, was also identified as a potential GDF. Yip3 catalysed the release of GDP from the GDI-Rab9 complex, enabling GTP binding and the subsequent insertion of Rab9 into the reconstituted liposomal membrane. Yip3 also did not enhance the intrinsic exchange of prenylated Rabs (Sivars et al., 2003). Evidence for other GDFs has been scant, however, and a consistent role in specific membrane targeting has also not been shown.

d) Guanine nucleotide exchange factors (GEFs):

Guanine nucleotide exchange may be another way by which GDP-bound Rab-GDI complexes are targeted to membranes. The subsequent GDP-GTP exchange catalysed by the GEF causes dissociation from GDI, as GDIs have reduced affinities for GTP-bound Rabs. Incidentally, it is contended that this would replace the need for GDF as an intermediate (Wu et al., 2010). Recent studies have supported the idea that GEFs are integral to the correct targeting of Rabs to membranes. Artificial targeting of the Rab5 GEF Rabex5 to the mitochondrial membrane resulted in a concomitant targeting of Rab5 from the endosome to the mitochondria. Reduction of the GEF activity of Rabex5 by targeted mutations to the VPS9 domain abolished this effect (Blumer et al., 2013). In another study, when Rab3GEP, the non-redundant GEF for Rab27a, was silenced, Rab27a was no longer targeted to melanosomes, but was instead mislocalised to perinuclear membranes. Rab3GEP is necessary but not sufficient for correct targeting, however, as other Rab27a mutants have been identified which retained the ability to bind Rab3GEP, but are still not correctly targeted (Tarafder et al., 2011).

If GEFs are indeed responsible for Rabs' membrane targeting, it then begs the question of how GEFs, which are also peripheral membrane proteins, are themselves

targeted to the specific membrane. One possibility is that these are membrane localised by a preceding Rab cascade. For example, the GEF for the late endosome-localised Rab7, the Hsp70-Hsp90 Organising Protein System (HOPS) complex, is initially localised to endosomal membranes because it interacts as an effector protein with Rab5, which precedes Rab7 on early endosomes (Rink et al., 2005). Another possibility is that the GEFs interact with other machineries located on the targeted membrane. For example, the Rab35 GEF connecdenn1 is recruited to endosomes that were formed from clathrin-mediated endocytosis via its interaction with AP2, the clathrin adaptor protein (Marat and McPherson, 2010). A third possibility is that the GEFs are themselves stabilised by a positive feedback loop. A typical example is that of the Rabex5-Rab5-Rabaptin5 complex, in which the Rab5 effector, Rabaptin5, stabilises the presence of the Rab5 GEF Rabex5 on endosomal membranes, enabling a synergistic association in a Rab5 microdomain on the endosome (Lippé et al., 2001).

e) Effectors:

The requirement for effector proteins in determining Rab subcellular localisation also appears to vary between Rabs. Rab27a mutants that are deficient in effector binding were still correctly localised to the melanosomes (Tarafder et al., 2011). The situation for Rab9, however, differs. Rab9, found on late endosomes, is required for the retrograde transport of mannose 6-phosphate receptor (M6PR) from the late endosome to the trans-Golgi network. TIP47, a Rab9 effector, has a high affinity for M6PR, and thus serves as a bridge between the Rab and its cargo. A chimera consisting of the Rab5 backbone and the Rab9 HV domain was found to bind both Rab5 and Rab9 effectors, and was found on the early endosome (the target membrane for Rab5). However, if TIP47 is overexpressed, the chimera moved to the late

endosome, highlighting the importance of the Rab9 effector interaction in determining its localisation, perhaps by creating a microdomain that enhances interactions through positive feedback (Aivazian et al., 2006). It is however of note that another Rab9 effector, p40, did not present the same effect. Also, depletion of TIP47 did not mislocalise Rab9. Together, the evidence suggests that there may be certain specific effectors that are important for localisation, but also that there may be redundancy in the system.

f) GTPase activating proteins (GAPs):

Although GAPs are, in a sense, the last in a series of interacting proteins that bind to a Rab in a Rab activation cycle, they also appear to be important in maintaining specific Rab domains on a membrane (Nottingham and Pfeffer, 2009). For example, Gyp1, an effector for yeast Rab Ypt32, acts as the GAP for the yeast Rab Ypt1. Both Ypt1 and Ypt32 are found on the Golgi membranes, with Ypt1 facilitating intra-Golgi transport and Ypt32 facilitating Golgi exit. As such, via Gyp1, Ypt32 is able to maintain a Ypt1-free zone, enabling each Rab to maintain a separate domain on the Golgi (Rivera-Molina and Novick, 2009).

1.1.3 Rab cascades and crosstalk between Rabs and their interacting proteins

A concept in membrane trafficking that is rapidly gaining experimental support and popularity is that of Rab cascades, referring to the progressive recruitment of Rab and/or Rab interacting proteins, such that a particular membrane compartment gradually takes on a different Rab protein from the initial Rab, and thus allowing a cascade of activated Rabs to carry out their function.

One classic example, already briefly described above, is that of the progression of Rab5 early endosomal compartments to Rab7 late endosomal compartments, thus facilitating the maturation from early to late endosome (Rink et al., 2005). Using a quantitative live cell imaging approach, the authors identified Rab5 early endosome compartments that gradually lost Rab5 and acquired Rab7. This maturation into late endosomes was mediated by the Class C VPS/HOPS complex, which is an effector for Rab5-GTP, and a GEF for Rab7 (Rink et al., 2005). A further layer of complexity was found in the *C. elegans* SAND1/Mon1, which was shown to displace Rabex5. This would ensure that the positive feedback associated with Rabex5-Rabaptin5 is terminated, thus enabling the endosome to mature to a Rab7-dontaining one. As SAND1/Mon1 recognises phosphotidyl inositol 3-phosphate (PI3P), it was postulated that SAND1/Mon1 binds only the more mature endosomes, which would have accumulated a large amount of PI3P via the action of phosphotidylinositol-3-kinase (PI3K), a Rab5 effector. In this way, Rabex5 is only displaced when the early endosome is ready to mature to a late endosome. SAND1/Mon1 also binds to Class C VPS/HOPS complex, enabling the recruitment of Rab7 (Poteryaev et al., 2010) .

Another example of a GEF being an effector of the preceding Rab in a cascade is the Rab22-Rabex5-Rab5 cascade (Zhu et al., 2009). Rabex5 consists of a GEF domain that contains a helical bundle and a VPS9 domain. Downstream of the GEF domain is the Rabaptin5 binding domain. Rabex5 also has an early endosomal targeting (EET) domain. Using a series of GST- affinity pulldown assays, Rabex5 was found to be an effector for Rab22, while serving as a GEF for Rab5. Rabex5 is recruited to Rab22 GTP-containing early endosomes via its EET domain. Rabex5 can then subsequently recruit Rab5. As an illustration of the complexity of the crosstalk

between various Rab-mediated endocytic pathways, it was found that overexpression of Rabex5 enhanced Rab5-mediated endosomal fusion but inhibited Rab22-mediated endosomal fusion, presumably because it competed with EEA1, the other Rab22 effector which is responsible for mediating fusion.

Sometimes, the same GEF can serve two consecutive Rabs, by recruiting a different subset of subunit proteins. The TRAnsport Protein Particle complex (TRAPP) in yeast is a multisubunit tethering complex on the Golgi that acts as a GEF for two different Rabs that function in two separate Golgi compartments. Ypt1 mediates ER-Golgi and intra-Golgi transport, while Ypt32 mediates exit from the Golgi. To ensure specificity of action, the TRAPP complex engages different subunits for the different steps. TRAPP I consists of 7 subunits while TRAPP II acquires an additional 3 subunits, thus switching the GEF specificity (Morozova et al., 2006).

Similarly, a divalent effector can bind to two consecutive Rabs. Rabenoysn5 was initially identified as a Rab5 effector, with a FYVE zinc finger domain (named after the cysteine rich Fab1, YOTB, Vac1, and EEA1 proteins). It was later shown to also bind Rab4-GTP in the same complex. In this way, Rab5 early endosomes can be channelled into the recycling pathway via recruitment of Rab4. Overexpression of Rabenosyn5 was shown to stimulate the fast recycling of the transferrin receptor (De Renzis et al., 2002).

These examples of Rab cascades highlight the complexity of Rab interactions and their regulation.

1.2 Overview of the Rab5 subfamily

Rabs have highly conserved domain motifs (Deneka et al., 2003). As with all small GTPases, Rabs contain two switch regions that change conformation upon GTP binding, enabling the engagement of various effector proteins. The five Rab family motifs are unique to the Rab family amongst the other small GTPases. Four regions, termed the subfamily regions, also have high homology within Rab subfamilies. The hypervariable region has the least sequence conservation (Fig. 1.4). These domain motifs are a means by which Rabs can be classified.

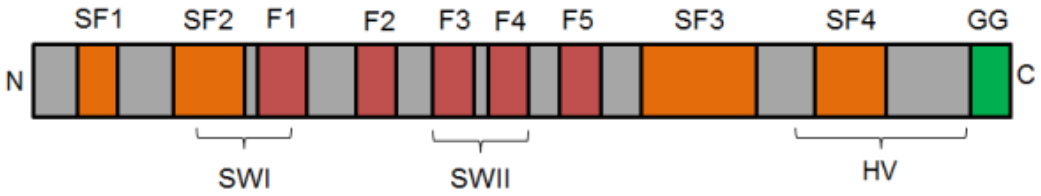


Fig. 1.4. Structural domains of Rab proteins
Domain motifs of Rabs which are highly conserved amongst the small GTPases. See text for more details. F: Family domain; SF: Subfamily domain; SW: Switch region; GG: prenylation domain; HV: Hypervariable domain.

The Rab5 subfamily of Rab proteins includes Rab5, Rab21, Rab22 and Rab31 (Pereira-Leal and Seabra, 2001). Members of this subfamily have been implicated in a variety of endocytosis-related trafficking steps, including that of cell surface receptors (Bucci et al., 1992; Simpson et al., 2004; Kauppi et al., 2002).

1.2.1 The endocytic system intersects with cellular signalling

As illustrated above, Rabs play important roles in membrane trafficking pathways within the cell, and the Rab5 subfamily is particularly important in the endocytic trafficking pathway. The endocytic system, in turn, is important in the control of cellular signalling pathways that are triggered by ligand binding to receptors, which sets off a cascade of signalling molecules (Di Fiore and De Camilli,

2001). Once ligand-bound, receptors can be endocytosed through the interaction between the tyrosine or di-leucine based motifs in their cytoplasmic tails with clathrin adaptor protein complex AP2 (Sorkin and von Zastrow, 2009). Ubiquitin also serves as a signal for receptor-mediated endocytosis. Ubiquitinated epidermal growth factor receptor (EGFR), for example, is recruited to clathrin coated pits via the ubiquitin-binding domains on Epsin, which itself mediates the interaction with clathrin (Sen et al., 2012). Ligand-receptor complexes that are internalised can be recycled or degraded. For example, the transferrin receptor or low-density lipoprotein receptor is recycled back to the cell surface, while receptors like EGFR or ligands like low-density lipoprotein are degraded. In this way, duration and strength of signalling is controlled. As such, Rab proteins such as those in the Rab5 subfamily can be expected to be important in the regulation of duration and strength of signalling.

Signalling activities can still persist while in endosomes. EGFR, for example, remains ligand-bound and phosphorylated while in endosomes. In an attempt to study the effect of endosomal signalling without input from plasma membrane signalling, the authors first treated cells with EGF in the presence of AG1478 and monensin, a tyrosine kinase inhibitor and inhibitor of receptor recycling respectively, which resulted in the internalisation of non-active ligand-bound EGFR. The inhibitors were then removed to allow signalling from endosomes to occur. The authors found that mitogen activated protein kinase (MAPK) continued to be phosphorylated by this process, but not phospholipase C (PLC) γ, which suggests that endosomal signalling differs somewhat from signalling at the plasma membrane, in which both MAPK and PLCγ become phosphorylated (Wang et al., 2002). As another example of signalling effects that occur when receptors are endocytosed, an endosomal pool containing Rab5 and its effector APPL1 (Adaptor protein, phosphotyrosine binding (PTB)

domain, pleckstrin homology (PH) domain, leucine zipper-containing protein 1) is also important for signalling that mediates cell proliferation. To elaborate, upon ligand activation of EGFR, PI3P accumulates on the early endosome. EEA1, which has PI3P-binding domains, is recruited to the early endosome and competes with APPL1 for Rab5-binding. As more EEA1 is recruited, APPL1 is eventually displaced from Rab5 (Zoncu et al., 2009) . Dissociated APPL1 translocates to the nucleus where associates with the nucleosomal remodelling and deacetylase complex (NuRD)/MeCP to mediate cell proliferation (Miaczynska et al., 2004). This is thus another example of how Rabs intersect with cellular signalling pathways.

While endocytosis mediates signalling, signalling can also mediate various aspects of endocytosis. For example, activation of EGFR leads to an increase in clathrin coated pit formation, by phosphorylating the clathrin heavy chain in a region that mediates the formation of the clathrin triskelion (Wilde et al., 1999). Also, tyrosine kinase activity of EGFR is believed to be important for Rab5 activation, through the recruitment of the RIN1, the Rab5 GEF, to the EGFR-bound Growth factor receptor-bound protein 2 (Grb2) and its downstream substrate p21Ras, which relieves the autoinhibition on RIN1 (Jozic et al., 2012).

1.2.2 Rab5 subfamily members and the EGFR trafficking pathway

Cell surface receptors are constantly endocytosed, both constitutively and when bound by its ligands. EGFR, for example, exists at steady state on the cell surface as a monomer. Upon binding of EGF or other ligands, the receptor dimerises and autophosphorylates, initiating a resultant signalling cascade by serving as a docking site for Src-homology 2 (SH2) or phosphotyrosineB (PTB) domain containing proteins (Yang et al., 2012). Examples of these include Grb2, PI3K, PLCγ,

and Epsin8. In the canonical pathway, Grb2 recruits Son of Sevenless, a GEF for p21Ras, via its Src-homology 3 (SH3) domains interacting with proline-rich sequences. Activated p21Ras facilitates the membrane recruitment and activation of Raf kinase, which triggers the Raf/MEK/MAPK pathway of cell signalling (Martinu et al., 2002). At the same time, ligand-bound EGFR is internalised from the plasma membrane and trafficked to early and late endosomes and eventually degraded in lysosomes, which results in a termination of its signalling (Sorkin and Goh, 2009; Ceresa, 2006). Alternatively, the receptor may be recycled to the cell surface (Masui et al., 1993). Different EGFR ligands trigger different endocytic sorting of the receptor. For example, EGF triggers both recycling and degradative pathways, whereas the transforming growth factor TGFα largely triggers recycling (Roepstorff et al., 2009). Many Rabs, including those of the Rab5 subfamily, have been implicated to varying extents in various trafficking steps of the EGFR internalisation pathway. For example, Rab5 (Huang et al., 2004) and Rab 21 (Simpson et al., 2004) have been shown to enhance the movement of EGFR from the cell surface into early endosomes, while Rab22 (the closest paralogue to Rab5 (Mishra et al., 2010)), as well as Rab21, have been implicated in the later trafficking steps, with a general role in recycling or terminating the EGFR signalling in late endosomes / lysosomes (Kauppi et al., 2002). In some studies, loss of Rab5 activity (either by siRNA or use of dominant negative Rab5 S34N) has also been shown to inhibit the exit of ligand-bound EGFR from the early endosome (Chen et al., 2009; Dinneen and Ceresa, 2004). The following section discusses these findings in more detail.

a) Rab5

Rab5 has 3 distinct isoforms (Rab5a, b and c) which share 80% homology. All serve to mediate early endosomal dynamics, but slight differences in tissue distribution, phosphorylation profiles etc. have been observed (Chiariello et al., 1999). Rab5a has been shown to be the main isoform required for EGFR trafficking. Silencing of Rab5a reduced the rate of degradation of EGFR in HeLa cells, while overexpression accelerated it (Chen et al., 2009). Only when all 3 isoforms of Rab5 were depleted was EGFR internalisation delayed (Huang et al., 2004; Chen et al., 2009), which led to the conclusion that Rab5 was more important for endosomal trafficking of EGFR than the initial internalisation of EGFR from the plasma membrane. EEA1, an effector of Rab5, was not displaced from early endosomal structures when Rab5 was silenced, suggesting that Rab5 alone was not essential for the localisation of EEA1 on early endosomes, and that the perturbation of EGFR trafficking seen was not due to a general disruption of endosomal dynamics (Chen et al., 2009). Silencing of Rab5 delayed the exit of EGFR from early endosomes. Of note is that it only delayed but did not completely block this exit, suggesting that the cell can still overcome the blockage, perhaps by compensatory mechanisms. RIN1 was the main GEF responsible for the observed Rab5 effect on EGFR trafficking (Chen et al., 2009). In another study, the dominant negative Rab5S34N did not perturb internalisation but affected the rate of degradation (Dinneen and Ceresa, 2004). There was now a delocalisation between Rab5 S34N positive endosomes and those containing EGFR, although internalisation of EGFR into endosomal pools still occurred (Dinneen and Ceresa, 2004). Again, this suggested that Rab5 played a more important role in EGFR trafficking than internalisation.

However, in other studies, Rab5 was also shown to have a role in EGFR internalisation from the cell surface. It was first found that in NR6 cells, dominant negative Rab5 S34N inhibited internalisation of ligand-bound EGFR (Barbieri et al., 2000). This was corroborated in HeLa cells, and injection of antibodies targeted to Rabaptin5, the Rab5 effector, also had the same effect (Chen and Wang, 2001). Also, the Rab5 GAP RN-tre was found to bind Grb2, and overexpression of RN-tre was found to inhibit internalisation of ligand-bound EGFR (Martinu et al., 2002). At the same time, activated Ras binds RIN1, releasing the effect of autoinhibition and enabling RIN1 to catalyse the GDP-GTP exchange on Rab5, enhancing receptor-mediated endocytosis (Tall et al., 2001). In contrast to the studies described in the previous paragraph, these studies suggest that Rab5 might also have a role in endocytosis of EGFR from the plasma membrane. The differences in observations might be due to the use of different cell types, which may differ in the localisation of exogenously expressed Rab5, and serves to highlight the variation in roles of Rab5 in EGFR trafficking. Incidentally, the studies by Martinu et al. (2002) and Tall et al. (2001) also provide evidence that receptors can recruit both GEFs and GAPs for a given GTPase. It is likely that the GEF and GAP work in concert to regulate the intricacies of EGFR internalisation and trafficking.

b) Rab22

Rab22 is localised on early endosomes and shares 52% sequence identity with Rab5. Silencing of Rab22 blocks the recycling of endocytosed cargoes like the transferrin receptor and major histocompatibility complex (MHC) I (Zhu et al., 2009). Rab22 is found on endosomes and interacts with EEA1 at its N-terminal region only (whereas Rab5 interacts with both the N and C-terminal region of EEA1). Dominant

active mutant Rab22 Q64L does not perturb internalisation of ligand-bound EGFR but does perturb its degradation. In fact, overexpression of wild-type, activating or inactivating mutants of Rab22 all decreased the degradation of EGF (Ceresa, 2006). This suggests that Rab22 needs to be recycled, perhaps because both the active and inactive form of Rab22 interact with specific proteins required for EGFR trafficking. A redistribution of various endocytic markers was also observed, leading the authors to suggest that the effect of Rab22 may be due, in part, to a general perturbation of the endocytic pathway. For example, it is also postulated that Rab22 facilitates interaction between TGN-derived membranes and the early endosome (Kauppi et al., 2002).

c) Rab21

Rab21 is predominantly localised to early endosomes (Egami and Araki, 2008). Cells overexpressing a Rab21 dominant negative mutant, Rab21 T33N, had defects in both internalisation of ligand-bound EGFR from the cell surface and subsequent trafficking from early to late endosomes (Simpson et al., 2004). Rab21 also plays a role in the internalisation of integrins from the cell surface. Rab21 binds directly to integrins via the α-tail, albeit in a nucleotide independent manner. p120RasGAP (which is not a GAP for Rab21) competes with Rab21 for integrin binding, and in this way regulates the presence of integrins in Rab21 endosomal compartments (Mai et al., 2011). More recently, Rab21 has also been shown to bind EGFR and overexpression of Rab21 enhances both unliganded and liganded EGFR internalisation from the cell surface, as analysed by confocal microscopic images, as well as degradation of unliganded EGFR, hence attenuating MAPK signalling (Yang et al., 2012). This is in contrast to the role of Rab5, which takes place only for ligand-bound EGFR, as EGF

stimulation has been shown to be important for activation of Rab5, by relieving the inhibition on RIN1 (Barbieri et al., 2000)

1.2.3 Other Rabs implicated in the EGFR trafficking pathway

Although not part of the Rab5 subfamily, it is worth mentioning some other Rabs that have also been implicated in EGFR trafficking (Ceresa, 2006). Rab4 and Rab11 have both been implicated in the endosomal recycling pathway. Expression of Rab4 dominant negative mutant Rab4 S22N resulted in a decrease in both recycling and degradation of EGFR, leading the authors to postulate that Rab4 is important for sorting in the early endosomal compartment (McCaffrey et al., 2001). Meanwhile, the Rab11 family interacting protein 2 (Rab11FIP2) has been shown to affect both receptor internalisation as well as recycling, leading the authors to suggest that the protein may be important for coupling internalisation to recycling of the EGFR (Cullis et al., 2002). Rab7 has been linked to the maturation of EGFR-containing late endosomes to lysosomes (Vanlandingham and Ceresa, 2009), as silencing of Rab7 blocks the exit of EGFR from the late endosome and reduces the rate of EGFR degradation (Ceresa and Bahr, 2006).

1.2.4 Introduction to Rab31

Rab31 was first cloned from human melanocyte cDNA and has been classified under the Rab5-related subfamily, based on sequence homology (Chen et al., 1996). Colleagues in the laboratory have previously reported that Rab31 is localised to the perinuclear region of the cell, colocalising with trans-Golgi network (TGN) markers such as TGN46 (Ng et al., 2007).

GAPex5 was first confirmed as a Rab31 GEF in studies looking at the insulin regulated trafficking of the glucose transporter Glut4. Although it can also serve as a GEF for Rab5, it has been shown to have a higher affinity for Rab31 (Lodhi et al., 2007). It contains an N-terminal Ras-GAP domain, a central PXXP sequence similar to other SH3 domain-binding sequences, and also a C-terminal VPS9 GEF domain for Rabs (Fig. 1.5 upper panel). The SH3 domain of Cdc42-interacting protein 4 (CIP4) was found to bind the PXXP sequence of GAPex5. The authors postulated that Glut4 in intracellular vesicles is normally held there in a futile cycle of fusion between vesicles that is mediated by active Rab31. When adipocytes are stimulated with insulin, CIP4 has been shown to translocate to the plasma membrane; GAPex5 thus translocates as well. As a result, the GAPex5 activation of Rab31 within the cell is lost. When Rab31 is no longer active, Glut4 is then freed from its futile cycle of fusion and released at the plasma membrane in response to insulin. In HeLa cells, GAPex5 has also been identified to bind to EGFR through Casitas B lineage lymphoma protein (Cbl), an E3 ubiquitin ligase, upon receptor dimerisation (Su et al., 2007). Cbl binds to phosphotyrosine motifs on ligand-bound EGFR and is responsible for the ubiquitination of EGFR, which serves as both an internalisation and degradation signal. GAPex5 interacts with Cbl via its Ras-GAP domain. RIN3 was identified more recently as another Rab31 GEF. It has an SH2 and proline-rich (PR) domain in the N-terminal region, as well as RIN-family homology (RH), VPS9, and Ras-association (RA) domains in the C-terminal region (Fig. 1.5 lower panel). It has higher GEF activity for Rab31 than for the other Rab5 subfamily proteins such as Rab5 and Rab22 (Kajiho et al., 2011). A specific role for RIN3 has not yet been found.

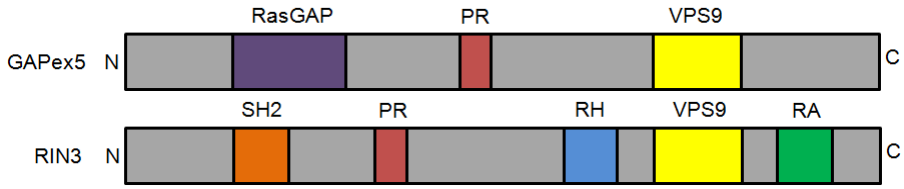


Fig. 1.5. Domains of Rab31 GEFs GAPex5 and RIN3
GAPex5 and RIN3 both contain the Vps9 GEF domain. See text for more details. PR: Proline-rich region; SH2: Src homology-2 domain; RH: RIN homology domain; RA: Ras association domain.

Currently identified putative effector proteins of Rab31 include the early endosome antigen 1 (EEA1) and the Adaptor protein, phosphotyrosine binding (PTB) domain, pleckstrin homology (PH) domain, leucine zipper-containing protein 2 (APPL2). EEA1 is a tethering factor that is recruited to early endosomes via PI3P through its C-terminal FYVE zinc finger domain. It forms a homodimer and mediates homo- and heterotypic fusion between endosomes. It has been identified as an effector protein for all the Rab5 subfamily members (Simpson et al., 2004; Kauppi et al., 2002; Simonsen et al., 1998; Lodhi et al., 2007). APPL2 contains an N-terminal Bin-amphiphysin-Rvs (BAR) domain, which is responsible for its curved structure that enables it to aid in membrane curvature. Adiponectin is a key hormone secreted mainly by adipose tissue that regulates several aspects of fatty acid and glucose metabolism (Yamauchi et al., 2003). Together with insulin, it stimulates the translocation of Glut4 to the cell surface and glucose uptake. APPL1 has been shown to interact with the adiponectin receptor to stimulate activity, and also with Akt and PI3K, two kinases involved in insulin signalling. APPL2 has been shown to be a negative regulator by competing with APPL1 for binding, or by sequestering APPL1 through heterodimer formation (Wang et al., 2009). Rab31 was identified to be a binding partner of APPL2 through yeast two-hybrid screening (King et al., 2012), although its physiological role in the process, if any, has yet to be characterised.

Currently, no GAPs have been identified for Rab31, nor have other GEFs or effectors been characterised, save for those mentioned above. There thus remains a dearth of knowledge about the interacting proteins of Rab31, and the potential roles they play in aiding Rab31 in mediating vesicular trafficking. As described in Chapter 1.1.3, Rab cascades have become of particular interest in the field. A better grasp of the interacting proteins of Rab31 would give some insight into the possible interplay between Rab31 and other Rabs, perhaps in mediating the 'handover' of cargo from one Rab-containing compartment to another. A few roles of Rab31 in membrane trafficking have currently been explored. As described above, Rab31 is postulated to play a role in the cycling of Glut4 in adipocytes (Lodhi et al., 2007). Rab31 was also found in oligodendrocytes (Rodriguez-Gabin et al., 2001) and shown in live imaging of HeLa cells to be found on M6PR-positive tubulovesicular structures emerging from the TGN (Rodriguez-Gabin et al., 2009). While the significance of this in oligodendrocytes has not yet been fully determined, it is postulated that the cycling of M6PR is important to the trafficking of myelin proteins. In support of this, independent of its conformational state, Rab31 was shown to interact with Oculocerebrorenal syndrome of Lowe protein (OCRL1), a protein which is mutated in Lowe syndrome, a disorder of the nervous system which includes degenerative demyelination (Rodriguez-Gabin et al., 2010). Although Rab31 is a member of the Rab5 subfamily, which have been implicated in endosomal trafficking steps, little is currently known about the role of Rab31 in endosomal trafficking. In Chapters 3 to 6 of this thesis, we describe our attempts to shed further insight on the role of Rab31 in trafficking and its physiological functions.

1.3 Physiological and pathophysiological activities of Rabs

Given the variety of ways in which Rabs have been implicated in cellular trafficking processes, it is not surprising that Rabs have been linked to many physiological and pathophysiological processes and diseases (Mitra et al., 2011). Five Rab genes have currently been associated with heritable monogenic diseases. Rab7 missense mutations underlie Charcot–Marie–Tooth type 2B neuropathy, a peripheral nervous system disorder that is believed to be due, in part, to the dysregulation of peripherin (a neuronal intermediate filament that has been shown to interact with Rab7) (Verhoeven et al., 2003; Cogli et al., 2013). Mutations in Rab18 (whose role in trafficking has not yet been clearly defined) cause Warburg micro-syndrome (Bem et al., 2011), a rare autosomal recessive genetic disorder characterized by microcephaly. Mutations in Rab23, which plays a role in the regulation of sonic hedgehog signalling, underlie Carpenter's syndrome, a rare autosomal recessive disorder associated with several congenital malformations (Jenkins et al., 2007; Ben-Salem et al., 2013). Rab27, which plays a role in melanosome transport via its effector myosin Va, has been implicated in Griscelli syndrome type 2 (Barral and Ramalho, 2002), a recessive disorder with pigmentation and immune defects. Mutations in Rab39B, a Golgi-localised neuronal Rab that may play a role in synaptic maintenance, are responsible for X-linked mental retardation (Giannandrea et al., 2010). In addition, Rab38 (mutated in the Ruby (red eyed dilution; R) locus of the rat and the homologous chocolate (cht) locus of the mouse) has been implicated in the autosomal recessive disorder Hermansky-Pudlak syndrome (HPS) (Oiso et al., 2004). Recently, mutations in the Rab38 GEF BLOC3 have also been identified, and it is postulated that the resulting defects in the biogenesis of lysosomal related organelles, of which Rab38 is believed

to play a role, gives rise to some of the symptoms observed in HPS, including albinism and impaired platelet function (Gerondopoulos et al., 2012).

The sections below focus briefly on two other Rab functions and activity which are highly relevant to the findings reported in this thesis.

1.3.1 Role of Rabs in cancer

Rabs have a variety of ways in which they can impact cancer and tumourigenesis (Recchi and Seabra, 2012; Chia and Tang, 2009). Conceivably, Rabs can mediate mechanisms which promote invasiveness, for example, by aiding in the secretion of matrix metalloproteinases (MMP), or by directing integrin trafficking, which aid in migration and invasiveness. Rab8, for example, was shown to enhance the exocytosis of membrane type 1 (MT1)-MMP (Bravo-Cordero et al., 2007). Rab25 was shown to coimmunoprecipitate the β1 subunit of α5β1 integrins. Rab25 and integrins were shown to colocalise at pseudopodal tips, and overexpression of Rab25 enhanced cell migration by maintaining an actively recycling pool of vesicles containing integrins near the cell surface (Caswell et al., 2007). In a situation of malignancy, Rab25 would increase the invasiveness of tumour cells. Consistent with this, in patients with ovarian cancer, Rab25 was shown to be amplified, and was found in higher levels in later stage tumours (Cheng et al., 2004).

Rabs also mediate several signalling pathways related to cell survival and proliferation. An example would be epidermal growth factor (EGF) signalling, which activates the downstream MAPK pathway. Rab5 was shown to enhance the endocytosis and degradation of ligand-bound EGFR. Surprisingly, this was translated into an enhanced MAPK phosphorylation. Also, Rab5 was found to be overexpressed in a majority of hepatocellular carcinomas (Fukui et al., 2007). It was shown that

activation of Rab5 promoted tumour progression by enhancing tumour migration and invasiveness via its interaction with focal adhesion complexes. By enhancing the internalisation of focal adhesion complexes, Rab5 aids in the rapid turnover required for focal adhesion assembly and disassembly (Mendoza et al., 2013).

Another way in which Rabs can influence tumour progression is by promoting drug resistance. For example, P-glycoprotein, an energy-dependent drug efflux pump, has been shown to be overexpressed in several multi-drug resistant human cancers. Rab4, which plays a role in recycling endosomes, interacts with the P-glycoprotein and was shown to reduce its surface expression (Ferrándiz-Huertas et al., 2011).

As such, targeting Rabs could, in the future, become an integral part of the arsenal used in cancer therapy, a concept which will be discussed in greater detail in Section 7.2.

1.3.2 Role of Rabs in the nervous system

The nervous system involves a complex network of trafficking processes, by virtue of the high rates of secretion of neurotransmitters and other signalling molecules from the presynaptic membrane and the internalisation of these signalling molecules at the post-synaptic membrane. As such, it is conceivable that Rabs would have a large impact on the functioning of the nervous system.

One way in which Rabs play a role is in the trafficking of postsynaptic neurotransmitter receptors. Glutamate receptors are synaptic receptors on the postsynaptic compartments of neuronal cells and receive glutamate as an excitatory neurotransmitter. Ionotropic glutamate receptors form a ligand-gated, non-selective cation channel that open in response to glutamate binding. The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) is one such example. The

movement of AMPARs in and out of the synaptic membrane modulate the strength of the synaptic response. Through a surface crosslinking assay, Rab5 overexpression was shown to mediate the internalisation and removal of AMPARs from the membrane, resulting in long-term depression which is important for synaptic plasticity. Long-term depression is believed to selectively weaken synapses in conjunction with long-term potentiation at other synapses to strengthen memory formation. Neurons expressing Rab5 dominant negative mutant Rab5 S34N failed to display long-term depression (Brown et al., 2005).

The proper cycling of synaptic vesicles, which carry neurotransmitters that are released at the presynaptic membranes and endocytosed at the postsynaptic membrane, also require Rabs. At least 11 Rabs have been found on synaptic vesicles (Pavlos and Jahn, 2011). Rab5, for example, was shown to colocalise with synaptophysin, a glycoprotein found on the synaptic vesicle (de Hoop et al., 1994). More recently, it was shown that mutations in Rabex5, the GEF for Rab5, resulted in defects in release of presynaptic neurotransmitters (Sann et al., 2012).

Rabs are also important to the regulation of neuronal-related growth factor signalling. For example, Rab22 was shown to have a role in nerve growth factor (NGF) signalling. Binding of NGF induces the internalisation of the NGF receptor TrkA into Rab22-containing endosomes. Endosomal signalling of internalised TrkA is responsible for neurite outgrowth, as TrkA on the plasma membrane, even if autophosphorylated, does not result in concomitant neurite outgrowth. Silencing of Rab22 inhibited the internalisation, with the resultant effect of reducing neurite outgrowth, in PC12 cells (Wang et al., 2011). This is in contrast to Rab5 and Rab21, which, when overexpressed, abrogates NGF signalling by enhancing the channelling

of endosomes to the degradative pathway. This example also helps illustrate the subtle differences between the roles of the various Rab5 subfamily members.

Rabs have also been found in non-neuronal cells in the nervous system such as the glial cells. There are three main types of glia – the astrocytes, which play a main role in the formation of the blood-brain barrier, oligodendrocytes which form the myelin sheath, and the microglia which act as immune cells in the otherwise immune-privileged central nervous system (CNS). Rab3 was found in astrocytes and oligodendrocytes (Madison et al., 1996), while Rab40C was found in oligodendrocytes (Rodriguez-Gabin et al., 2004). As yet, the exact functions of these Rabs in the neural system are largely unknown (Ng and Tang, 2008). It is conceivable that in oligodendrocytes, Rabs play a role in regulating myelination. In astrocytes, vesicular trafficking is important to, among other things, the exocytosis of neuropeptides that regulate cerebral blood flow, and the regulation of glutamate transporter density at the plasma membrane which help to regulate the strength of synaptic signalling (Kreft et al., 2009).

1.4 Rationale for studies reported in this thesis

In our lab, we have found Rab31 to be localised to the perinuclear region of the cell, colocalising with trans-Golgi network (TGN) markers such as TGN46 (Ng et al., 2007). The TGN-localised staining persisted even in cells that overexpressed Rab31, indicating the fidelity of Rab31's TGN localisation. The first part of this thesis therefore explores possible mechanisms by which Rab31 is localised.

We subsequently noted that Rab31 not only localises to the TGN, but also, in part, to the endosomal network. Both are major focal points for many vesicular transport pathways in the cell (Gu et al., 2001). Rab31 is therefore situated at a crossroad of many trafficking steps. Rab31 has been shown to function in Golgi-endosome trafficking of mannose 6-phosphate receptor (Rodriguez-Gabin et al., 2001; Rodriguez-Gabin et al., 2009), but has not been directly implicated in endocytic trafficking pathways. However, it is structurally grouped as a member of the Rab5 subfamily, and it also shares similar GEFs and effectors with other Rab5 subfamily members, notably GAPex5 and EEA1 (Lodhi et al., 2007; Mishra et al., 2010). GAPex5 contains a Ras-GAP domain and also a GEF domain for Rabs, and has been identified to bind to EGFR through Cbl, an E3 ubiquitin ligase, upon receptor dimerisation (Su et al., 2007). EEA1 participates in endosomal tethering and docking (Mills et al., 2001). Given the above possible connections, we investigated how Rab31 would affect the trafficking of EGFR, after stimulation with EGF (ligand-bound EGFR). This constitutes the second part of this project. Our studies were performed in A431 cells, a human epidermoid carcinoma line that has high levels of EGFR expression, and subsequently repeated in HeLa cells, as a second cell line to show that the phenomena were not specific only to A431. Because endogenous Rab31 levels are low, the cell lines were made to stably express Rab31.

Tissue expression pattern survey in mice revealed Rab31 to be enriched in the adult brain. Currently, the reason for the enrichment of Rab31 in the brain, and its physiological roles there, are not known. By co-staining with various markers for the different cell types in the brain, we found Rab31 to be enriched in astrocytes rather than in oligodendrocytes or neurons. Moreover, in a survey of the mouse embryonic brain, we found Rab31 to be enriched in radial glia cells (Ng et al., 2009). Radial glia develop into the various progenitor cells that will eventually give rise to the three main cell types of the brain. As the animal matures from embryonic stages to the adult stage, a population of radial glia cells appear to retain their neurogenic potential in the adult brain and become the neural stem cells of the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus, the two main areas of neurogenesis in the adult. Given Rab31's presence in both GFAP-positive cells in the adult brain and radial glia cells in the embryonic brain, we sought to determine what role Rab31 might possibly play in the brain, especially in light of the role of these cells in neurogenesis. This investigation was further fuelled by our findings from the second part of this study, that Rab31 plays a role in the trafficking of EGFR, which is an important signalling component in neurogenesis and differentiation. The third part of the project therefore looks at the physiological role of Rab31 in neural progenitor cell differentiation.

2. Materials and Methods

2.1 Gene constructs

Human Rab31 (IMAGE clone: 4792577), Rab22 (IMAGE clone: 3907891), GAPex5 (IMAGE clone: 40083103) and RIN3 (IMAGE clone: 30332851) expressed-sequence tags were obtained (Research Instruments, Singapore). Mouse Rab31 was kindly provided by Prof Mitsunori Fukuda (Tohoku University, Japan). pmCherryC1-Rab5a from the laboratory of Prof Christien Merrifield (National Centre for Scientific Research, France) was obtained from Addgene (Cambridge, MA, USA) (Plasmid No. 27679). pCI-Neo Rab11 was constructed from Rab11 cDNA kindly provided by Prof Mary McCaffrey (University College Cork, Ireland). Thu dominant negative mutant Rab31 S19N was generated by Dr Ng Ee Ling (National University of Singapore, Singapore) using nested primers carrying the mutation (Ng et al., 2007).

The primers below were used to generate the following Rab31 mutants:

Rab31 mutant lacking hypervariable region (amino acids 165-192):
Forward: 5' CTC GAATTC A ATG ATG GCG ATA CGG GAG CTC 3' Reverse: 5' CTT GTC GAC TCA ACA GCA GCG GCT GAT TCC TTG AAA GAG CTC TTC GAT ATT AGC 3'
Rab31 mutant lacking prenylation region (C terminal cysteines amino acid 193-194):
Forward: 5' CTC GAATTC A ATG ATG GCG ATA CGG GAG CTC 3' Reverse: 5' CCT GTC GAC TCA GGC TTG CAT GGT TGG CTT CTC AAC TTT GAT TGT TCC 3'

Table. 2.1. Primers used to generate various Rab31 mutants

Constructs were inserted into various vector backbones, including pCI-Neo (Promega, Madison, WI, USA), pmCherry-C1 (Clontech, CA, USA), pEGFP-C1 (Clontech) and pGEX-4T-3 (GE Healthcare, Singapore). Plasmid maps are shown in Appendix 1.

2.2 Antibodies

Rabbit anti-sera against Rab31 was generated by repeated immunization of New Zealand white rabbits with glutathione-S-transferase (GST) fused to the C-terminal 37 amino acids of human Rab31. Mouse monoclonal antibody against Rab31 was obtained from Abmart (Shanghai, China). The following commercial primary antibodies were used: neuronal marker, bIII-tubulin (TuJ) (Research Diagnostics, Flanders, NJ, USA), oligodendrocyte marker, 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) (Abcam, Cambridge, UK), astrocyte marker, glial fibrillary acidic protein (GFAP) (Sigma–Aldrich Pte. Ltd., Singapore), progenitor cell marker, nestin (Chemicon International, Inc., Temecula, CA, USA), proliferating cell nuclear antigen (PCNA) (Abcam), immature neuronal marker, doublecortin (DCX) (Santa Cruz Biotechnologies, Dallas, TX, USA), EGFR (Merck Millipore, Singapore), the cation-independent M6PR (Abcam), early endosome antigen 1 (EEA1) (BD Biosciences, San Jose, CA, USA), GAPex-5 rabbit polyclonal antibody (Abcam), RIN3 (Abnova, Taipei, Taiwan), the trans-Golgi marker TGN46 (AbD Serotec, Kidlington, UK), cis-Golgi marker GM130 (BD Transduction Laboratories, San Jose, CA, USA), Rab11 (Abcam), Rab5 (BD Transduction Laboratories) and γ-tubulin (Sigma). The CD63 hybridoma H5C6 monoclonal antibody developed by J.T. August and J.E.K. Hildreth was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. The following secondary antibodies were used for western blotting: horse radish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit antibodies (All Eights Pte. Ltd., Singapore). The following secondary antibodies were used for immunofluorescence: Fluorescein isothiocyanate (FITC), Texas-Red (TxR) or Cy5-conjugated anti-mouse, anti-rabbit, anti-sheep and

anti-goat antibodies (All Eights Pte. Ltd., Singapore). Dilutions of primary antibodies used for Western blots are 1:1000 in 3% bovine serum albumin (BSA) in PBS-T (0.05% Tween-20 in phosphate buffered solution (PBS)). Secondary antibodies are used at 1:5000 in 5% skimmed milk (Anlene) in PBS-T. For immunofluorescence studies primary antibodies are used at 1:50 to 1:100 dilution, and secondary antibodies at 1:100 dilution, in blocking buffer consisting of 5% fetal bovine serum (FBS) and 2% BSA in PBS.

2.3 Cell culture and transfection

A431 and HeLa cells (ATCC) were typically seeded at 5 x 10^4 cells/cm^2 density in high-glucose Dulbecco Modified Eagle medium (DMEM) (Hyclone, Logan, UT, USA) supplemented with 10% heat-inactivated FBS (Hyclone), 1% antibiotic/antimycotic (Invitrogen, Carlsbad, CA, USA) and 1 mM sodium pyruvate (Invitrogen). Transfection was performed with Lipofectamine 2000 (Invitrogen) or XtremeGENE HP DNA transfection reagent (Roche Diagnostics, Alameda, CA, USA) according to the manufacturer's protocols. Stably transfected cells were selected with 1.2 mg/ml G418 (Invitrogen), and single-cell cloned.

2.4 Primary mouse neural progenitor cell (NPC) culture

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and animals were treated in accordance with the IACUC guidelines in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved animal facility. NPC isolation was performed from established protocols with modifications (Chojnacki and Weiss, 2008; Low et al., 2012). In brief, brains of E15 embryos were harvested from pregnant C57/BL6 mouse. The ganglionic eminence

was dissected from the brains, dissociated, and cultured in suspension in culture media comprising Dulbecco's Modified Eagle Medium/nutrient mixture F-12 (DMEM/F12) (Hyclone), 0.66% glucose, 2 mM glutamine, 14.6 mM NaHCO3, 5 mM HEPES buffer, 23 μg/ml insulin, 93 μg/ml transferrin, 19 nM progesterone, 56 nM putrescine, 21 nM sodium selenite, 2% B27 (Invitrogen), 5 ng/ml fibroblast growth factor (FGF)-2 (Peprotech, Rocky Hill, NJ, USA) and 20 ng/ml EGF (Peprotech). After 5 days, the neurospheres formed in culture were dissociated into single cells using Accutase (Invitrogen) and plated at 2.5 x 10^4 cells/cm^2 density on poly-D-lysine (PDL) and laminin (Sigma) coated plates or coverslips, and maintained in DMEM/F12 supplemented by 1% N2 supplement (Invitrogen), 1% penicillin/streptomycin (Invitrogen) and 1 mM l-glutamine (Invitrogen), FGF-2 and EGF, both at 20 ng/mL and 5 μg/mL heparin (Merck). Cells were induced to differentiate by culturing for 5 days in different cell culture medium. Astrocyte differentiation medium consisted of DMEM/F12 supplemented with L-glutamine, N2 supplement, 1% FBS (Hyclone) and 10 ng/mL platelet derived growth factor-bb (PDGFbb) (Peprotech). Neuronal differentiation medium consisted of DMEM/F12 supplemented with L-glutamine, N2 supplement, B27 supplement and 5 ng/mL FGF-2.

2.5 Expression silencing

siRNA-mediated silencing was carried out by Lipofectamine RNAiMAX (Invitrogen)-mediated transfection of two 27-mer RNA duplexes (Integrated DNA Technologies, Inc., Coralville, IA, USA), according to the manufacturer's protocols. siRNA were generated according to the following sequences:

Human Rab31:	5'-rGrGrArArTrArCrGrCrTrGrArArTrCrCrArTrArGrGrTrGCC-3' 5'-rGrTrGrCrCrTrTrGrTrGrGrArArArTrGrArArCrTrTrCrACA-3'
Mouse Rab31:	5'-rGrGrArGrTrArCrGrCrTrGrArArTrCrCrArTrArGrGrTrGCC-3' 5'-rGrTrArCrTrArCrCrGrArGrGrArTrCrTrGrCrTrGrCrArGCC-3'
EEA1:	5'- rGrCrArGrGrArUrUrCrArGrCrArArArGrArArArArGrArArCAG-3' 5'-rGrUrArUrGrArUrGrArArGrArArArGrGrArGrUrCrUrUrCGA-3'
APPL2	5'-rCrCrArCrCrArCrCrUrGrGrGrArGrArGrGrCrUrUrUrArUTT-3'
GAPex5:	5'-rArArGrArArUrCrGrArUrUrArCrCrUrArUrArGrCrArArCUC-3' 5'-rGrCrArGrGrArGrGrArGrCrGrUrCrUrGrCrArArGrArArCTG-3'
RIN3:	5'-rCrGrArCrCrArGrCrCrArCrCrUrCrUrUrGrGrArArArUrUGC-3' 5'-rCrCrArCrCrArCrUrGrArCrCrUrArGrGrUrGrUrGrArCrCAC-3'
Rab5a:	5' – rGrUrArCrUrArCrArGrArGrGrArGrCrArCrArArGrCrArGCC -3' 5' – rCrCrCrArCrArCrArArArCrCrArArCrCrArGrGrArArUrCrAGT- 3'

Table. 2.2. siRNA designed for silencing experiments

Rab31 depletion was also performed using HuSH 29-mer shRNA (Origene, Rockville, MD, USA) transfected into cells.

2.6 Retroviral transduction

Engineered murine retroviruses were made to express GFP along with the shRNA. GFP expression was under the control of EF1α promoter and shRNA against Rab31 was co-expressed under the control of human U6 promoter in the same vector, kindly provided by Dr. Eyleen Goh and Ms. Heidi Liou (Duke-NUS Graduate Medical School, Singapore). Engineered retroviruses were produced by co-transfection of retroviral vectors and vesicular stomatitis virus G glycoprotein (VSVG) into HEK293gp cells, collected from the supernatant by ultracentrifugation and

resuspended in PBS. NPCs were plated together with retroviruses and assessed for GFP expression and knockdown after 2 days.

2.7 Reverse-transcription and real-time PCR

Total RNA was harvested from approximately 2 x 10^6 cells using Qiagen RNA isolation kit (Qiagen, Singapore). One-step RT-PCR was performed using Qiagen RT-PCR kit (Qiagen) on 0.5 μg total mRNA. The following thermal cycling parameters were used: 50°C at 30 min for reverse transcription, and 95°C at 15 min for activation of DNA polymerase. This was followed by 35 cycles of PCR: 94°C at 1 min for denaturation of template, 60°C at 1 min for annealing, and 72°C at 1 min for extension. The products were run on a 1% agarose gel and visualised.

For real-time PCR, cDNA was synthesised from total RNA using random hexamers from the TaqMan Reverse Transcription Reagent (Applied Biosystems) according to the manufacturer's protocols. The following thermal cycler parameters were used: 25˚C for 10 min to allow annealing, 48˚C for 40 min for extension of first strand cDNA synthesis, 95˚C for 5 min for inactivation of reverse transcriptase, then held at 25˚C. Real-time PCR with SYBR green detection (Applied Biosystems) was performed using ABI Prism 7000 Sequence Detection System (Applied Biosystems). Appropriate non-template controls were included and dissociation analysis was performed at the end of each run to confirm the specificity of the reaction. The following thermal cycling parameters were used: 95˚C for 10 min, for activation of AmpliTaq Gold DNA Polymerase, followed by 40 PCR cycles of 95˚C for 15 sec for denaturation and 60˚C for 1 min for annealing and extension. The threshold cycle (C_T), the cycle number at which the change in the fluorescence of the reporter dye crossed

the threshold, was set automatically by the software. Expression levels compared to the 0 hour were analysed using the $2^{-\Delta\Delta CT}$ method.

The following primers used for amplification are shown in Table. 2.3. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was used as the reference gene.

Rab5a	Forward: 5' CGC GAA TTC ATG GCT AGT CGA GGC GC 3' Reverse: 5' CCT GTC GAC TTA GTT ACT ACA ACA CTG ATT CC 3'
Human Rab31	Forward: 5' CTC GAATTC A ATG ATG GCG ATA CGG GAG CTC 3' Reverse: 5' TCG GTCGAC TCA ACA GCA CCG GCG GCT 3'
Mouse Rab31	Forward: 5' CAC TAA GCA GGA TTC ATT TCA TAC C 3' Reverse: 5' ATC AGA GAG GTC ACA CTT GTT C'
GAPex5	Forward: 5' GTC GAA TTC ATG GTG AAA CTA GAT ATT CAT ACT CTG 3' Reverse: 5' CCT CCC ATC AAC AAA TTG TGT ATC TTC 3'
RIN3	Forward: 5' GCG GAA TTC A ATG ATC CGA CAC GCC GGG GCG 3' Reverse: 5' CTA GCC ACC ACC CGG TGC AGG ATC 3'
GFAP	Forward: 5'AAC AAC CTG GCT GCG TAT AG 3' Reverse: 5' TCT CGA ACT TCC TCC TCA TAG AT 3'
Nestin	Forward: 5'AGA AGC AGG GTC TAC AGA GT 3' Reverse: 5' TCC AGC AGA GTC CTG TAT GTA 3'
G3PDH	Forward: 5' ATC TTC CAG GAG CGA GAT CC 3' Reverse: 5' AGA GGG GGC AGA GAT GAT GA 3'

Table. 2.3. Primers designed for PCR.

2.8 EGF pulse-chase experiments

Cells were serum starved overnight in basal DMEM. 0.25 µg/mL to 0.5 µg/mL TxR or FITC-tagged epidermal growth factor (EGF) (Molecular Probes, Invitrogen Corp. (Carlsbad, CA, USA) or non-conjugated EGF (Peprotech) was incubated with cells on ice for 20 min, followed by a 5 min incubation at 37°C for internalisation. Cells were then acid washed (150mM NaCl, 50mM glycine) before being returned into complete DMEM at 37°C for chase and fixed at various time points or harvested for cell lysate.

2.9 Collection of cell lysate and Western blot

For Western immunoblot analysis, tissue from Sprague-Dawley rat or cells from mammalian cell culture were lysed with lysis buffer (50 mM Tris pH8, 1 mM EDTA pH8, 150 mM NaCl, and 1% Triton X-100 with protease inhibitor cocktail (Roche) for 60 min. Lysates were then subjected to reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), electroblotted onto Hybond C-extra (Amersham Biosciences, UK) nitrocellulose membranes and probed with the respective antibodies. Band densities were quantified using Image J (US NIH, Bethesda, MD, USA), as a means of calculating the relative protein levels present.

2.10 Immunocytochemistry, immunohistochemistry, and immunofluorescence microscopy

For immunohistochemistry, 4% paraformaldehyde-perfused embryonic and postnatal mice brain tissue were optimal cutting temperature (OCT) (Electron Microscopy Sciences, Hatfield, PA, USA)-embedded and sectioned by cryostat at 20 μm thickness (Leica Microsystems, Wetzlar, Germany). Fluorescence labelling of tissue cryosection was performed with various antibodies and visualised with Carl Zeiss 710 (Oberkochen, Germany) confocal imaging system. For immunofluorescence microscopy, cells plated on cover slips and subjected to various treatments were fixed with 4% paraformaldehyde followed by sequential incubation with the primary and secondary antibodies. Fluorescence labelling was visualized using the Carl Zeiss 710 confocal imaging system. Images were collected in separate z sections and final images presented are typically from collapsed z stacks. Image processing was done Carl Zeiss' using Zen 2010 software. Particle size was measured using Image J.

2.11 Live-cell imaging

Cells expressing EGFP-Rab31 were grown on 35 mm glass-bottomed dishes (Ibidi, Germany) and treated with EGF-TxR as described above. Timelapse imaging on a single plane was performed at various intervals using LSM710 confocal microscope (Carl Zeiss, Germany), at a scanning speed of 2.5s per frame.

2.12 Flow cytometry

Cells were collected and fixed for 15 min in 2% paraformaldehyde. 1 x 10^5 cells were resuspended in blocking/incubation buffer (0.5% bovine serum albumin (BSA) in PBS) for 10 min, followed by subsequent incubation with FITC-labelled anti-EGFR antibodies (Cell Signalling Technology, Beverly, MA, USA) at 4°C, overnight. 1 x 10^4 cells were analysed using BD FACSCanto II Flow Cytometer (BD Biosciences) and histogram displaying count of fluoresecent intensity was presented using Flowing Software 2 (Turku Centre for Biotechnology, Finland).

2.13 Glycerol gradient sedimentation

Samples were lysed with lysis buffer containing 10 mM HEPES, 2 mM MgCl2, 10 mM KCl, 0.5 mM EDTA, 150 mM NaCl and 1% TritonX-100. Samples were treated with or without GTPγS (Merck Millipore) and diluted to a final solution containing 0.5% TritonX-100. 2 mg of lysate was loaded with onto 11 mL 5-45% glycerol gradients. Centrifugation was for 18 h at 38 000 r.p.m.in a Beckman SW41Ti rotor maintained at 4°C. 1 mL Fractions were collected and proteins were precipitated in 20% trichloroacetic acid (TCA), followed by cold acetone wash. Standard protein markers were applied to a glycerol gradient and run in parallel.

2.14 Co-immunoprecipitation

To analyse interacting partners of Rab31, 2 µg of Rab31 antibody was incubated at 4ºC overnight with 1 mg cell lysate loaded with 1 mM GTPγS at room temperature for 20 min, then bound to 50 µl Protein A beads (GE Healthcare, Singapore). After incubation, beads were washed with ten column volumes of lysis buffer. Elution of bound proteins was performed with loading buffer at 72ºC. Eluted proteins were analysed by SDS–PAGE and Western blot.

2.15 GST affinity pulldown assay

GST-fusion proteins of the Rabs were expressed in *Escherichia coli* DH5α or BL21-DE3 cells and purified by standard protocols. In brief, cells were harvested by centrifugation, resuspended and sonicated. The supernatant was incubated with glutathione beads (GE Healthcare) and eluted in elution buffer consisting of 50 mM Tris pH8, 0.1% TritonX-100, and 20 mM glutathione. To perform affinity pull-down of interacting partners, GST–Rab31 was added to 1 mg cell lysate and loaded with 1 mM GTPγS at room temperature for 20 min, then bound to 50 µl glutathione beads overnight at 4°C. After incubation, beads were washed with ten column volumes of lysis buffer. Elution of bound proteins was performed with loading buffer at 72°C.

2.16 Statistical analysis

Images and data presented in this thesis are typically representative of at least 3 independent experiments. Statistical analysis was performed using unpaired Student's t-test and one-way analysis of variance (ANOVA) as appropriate. Data shown in bar graphs usually represent means of 3 independent experiments assayed in triplicate. Error bars indicate standard error of means (SEM) of 3 independent experiments.

3. Domains and interactions responsible for the subcellular localisation of Rab31

3.1 Chapter Introduction: Localisation of Rab proteins to distinct membranes

As discussed in Section 1.1.2, a variety of mechanisms exist which aid in the localisation of Rabs to specific membranes. Newly synthesised Rabs are post-translationally modified by prenylation (Pereira-Leal et al., 2001), after which Rabs can be inserted into membranes. At the membranes, Rabs are activated by GEF-mediated GDP-GTP exchange, and engage effector proteins. GAPs enhance the GTPase activity of the Rabs, thus inactivating them (Nottingham and Pfeffer, 2009). GDP-bound Rabs can be extracted from the membrane by GDP dissociation inhibitors (GDI) (Ullrich et al., 1993; Ullrich et al., 1994; Soldati et al., 1994). Several mechanisms, based both on Rab structure and Rab interacting proteins, participate in targeting Rabs to their specific membranes / organelles. Structural domains such as the Rab hypervariable domain and the prenylation domain have been shown to be important for membrane association. Interacting proteins such as GDIs and GDI displacements factors (GDF) have also been shown, in some cases, to carry information on subcellular membrane specificity. Activating proteins, such as GEFs, may also be responsible for the specific membrane localisation of some Rabs, while effector proteins may also interact with Rabs and hold them in specific microdomains. Lastly, inactivating proteins such as GAPs may work to negatively regulate Rabs and delineate membrane regions which Rabs cannot be present.

As a whole, there therefore appears to be a wide variety of mechanisms for targeting Rabs to their specific membranes, which may vary for different Rabs. In the course of our experiments, we have observed that Rab31 appeared to be rather faithfully targeted to the trans-Golgi network (TGN) even when transiently overexpressed in cultured mammalian cells (see for example, Fig. 3.3). We thus

sought to identify which mechanisms might be important for this specific targeting of Rab31.

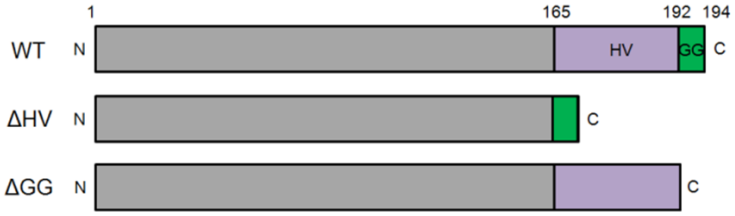
3.2 Results: Dependence of Rab31 subcellular localisation on functional domains

To identify regions within Rab31 that might be responsible for its subcellular localisation, we created Rab31 mutant constructs lacking either the hypervariable domain (ΔHV) or the prenylation domain (ΔGG) (Fig. 3.1A and B). We first sought to identify if the HV region of Rab31 was important for Rab31 membrane localisation. Since Rab22, a close homologue of Rab31, although almost identical in sequence to the latter except for the HV region (Fig. 3.1C) (Chen et al., 1996), is localised to endosomes rather than the TGN, we rationalised that the HV region might contain information about the TGN specific localisation of Rab31. While wild-type Rab31 strongly colocalised with the TGN marker TGN46 (Fig. 3.2 first panel) in A431 cells, we found that the Rab31 mutant, Rab31 ΔHV, was visibly cytosolic with some nuclear staining (Fig. 3.2 second panel). This is in line with observations by Chavrier et al. (1991), and suggests perhaps that for the Rab5 subfamily, the HV region is important for correct protein folding and post-translational modification for membrane insertion. Further biochemical studies to investigate if Rab31 ΔHV was correctly prenylated would serve to shed further light on this.

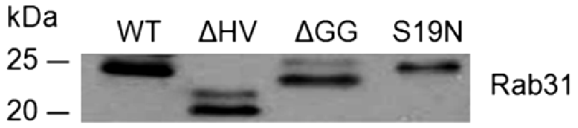
We next sought to identify if the C-terminal cysteine residues for prenylation was important for Rab31 membrane localisation. We rationalised that if interactions with GEFs or effector proteins were responsible for targeting and holding Rab31 in a microdomain on the TGN, soluble Rab31 might still be found associated with the TGN membrane despite its lack of prenylation. We created a Rab31 truncation mutant lacking the two C-terminal cysteine residues. We found that the Rab31 mutant, Rab31

ΔGG, was cytosolic (Fig. 3.2 third panel). This suggests that interactions with TGN-localised GEFs or effectors, if any, were not sufficient to hold Rab31 at the TGN. This was corroborated by looking at the localisation of the dominant negative Rab31 S19N mutant, which is unable to exchange GDP for GTP. As such, the S19N mutant can be expected to engage its GEF without releasing it. We found that Rab31 S19N, like Rab31 ΔGG, was also cytosolic (Fig. 3.2 fourth panel), suggesting that interaction with GEF proteins is not sufficient to localise Rab31 to the TGN. Prenylation of Rab31 may still be critical for membrane localisation, perhaps because Rab31 must first be inserted into membranes before functional association with interacting proteins can occur.

A)



B)



C)

Query: Rab31 [Homo sapiens]
Sbjct: Rab22 [Homo sapiens]

```
                                                    SWI
Query  1    MAIRELKVCLLGDTGVGKSSIVCRFVQDHFDHNISPTIGASFMTKTVPCGNELHKFLIWD  60
            MA+RELKVCLLGDTGVGKSSIV RFV+D FD NI+PTIGASFMTKTV   NELHKFLIWD
Sbjct  1    MALRELKVCLLGDTGVGKSSIVWRFVEDSFDPNINPTIGASFMTKTVQYQNELHKFLIWD  60
                SWII
Query  61   TAGQERFHSLAPMYYRGSAAAVIVYDITKQDSFYTLKKWVKELKEHGPENIVMAIAGNKC  120
            TAGQERF +LAPMYYRGSAAA+IVYDITK+++F TLK WVKEL++HGP NIV+AIAGNKC
Sbjct  61   TAGQERFRALAPMYYRGSAAAIIVYDITKEETFSTLKNWVKELRQHGPPNIVVAIAGNKC  120

Query  121  DLSDIREVPLKDAKEYAESIGAIVVETSAKNAINIEELFQGISRQIPPLDPHENGNNGTI  180
            DL D+REV  +DAK+YA+SI AI VETSAKNAINI ELF  ISR+IP  D +
Sbjct  121  DLIDVREVMERDAKDYADSIHAIFVETSAKNAININELFIEISRRIPSTDANLPSGGKGF  180

Query  181  KVEKPTMQASRRCC  194
            K+ +   +  R CC
Sbjct  181  KLRRQPSEPKRSCC  194
```

Fig. 3.1. Rab31 and its mutants
A) Rab31 mutant constructs were expressed in pDMyc expression vector (see Appendix 1 for vector map).
B) A431 cells were transfected with the various Rab31 mutant constructs as indicated and the wild type and various mutant proteins were analysed after 48 h by SDS-PAGE and Western blot using anti-Myc antibodies. WT: Rab31 wild type protein; ΔHV: Rab31 mutant lacking the HV domain (amino acids 165-192); ΔGG: Rab31 mutant lacking the prenylation domain (Cysteines 193 and 194); S19N: Rab31 dominant negative mutant incapable of GDP-GTP exchange.
C) Amino acid sequence alignment of Rab31 and Rab22
Amino acid sequences of *Homo sapiens* Rab31 and Rab22 were aligned using NCBI's Blast program. SWI: Switch I region; SWII: Switch II region. Green box highlights region of highest sequence variability, which is the hypervariable (HV) domain.

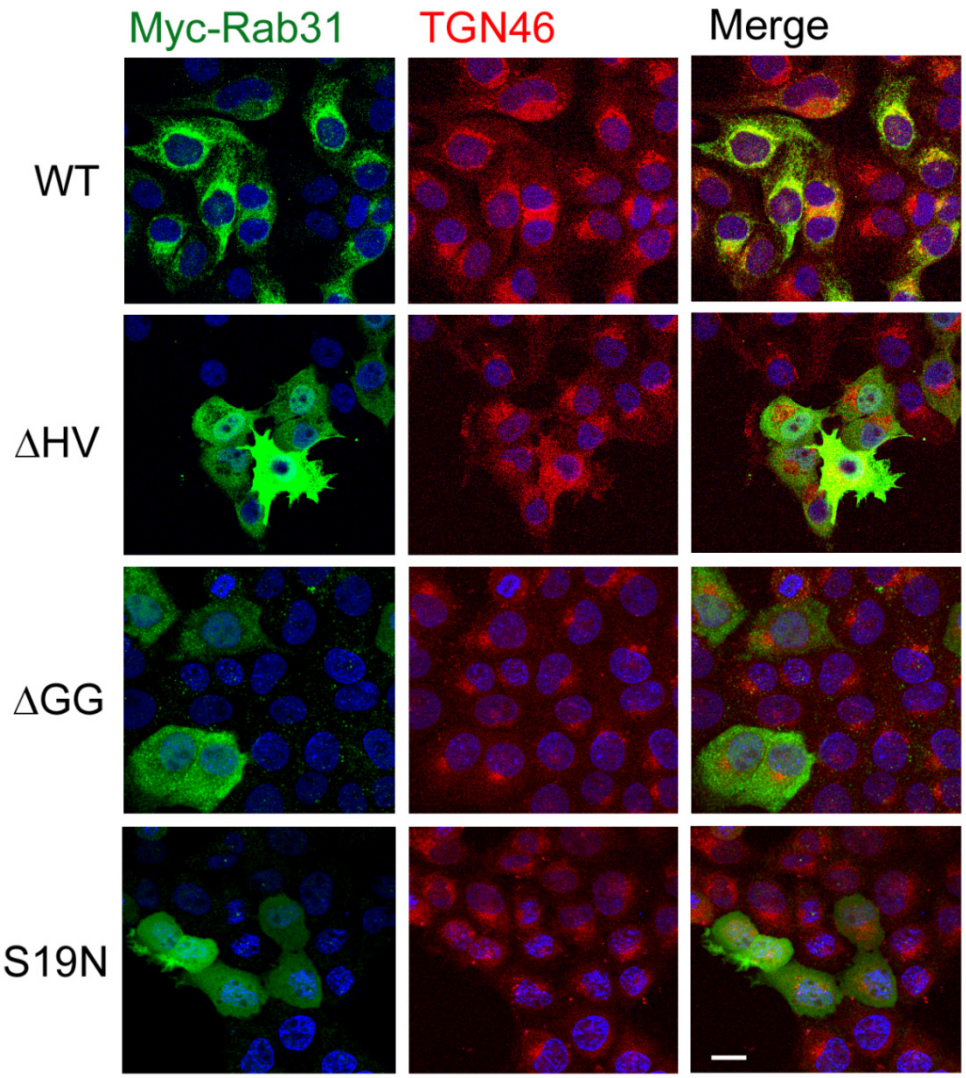


Fig. 3.2. Subcellular localisation of Rab31 and its mutants
A431 cells were transfected with the various Rab31 mutant constructs as indicated and observed after 48 h. Cells were fixed and immunostained for Myc-tagged Rab31 (green) and TGN46 (red). Nuclei are visualised with Hoechst 33342 stain. Scale bar = 20 μm.

3.3 Results: Dependence of Rab31 subcellular localisation on interacting proteins

Dependence on GEFs

Although the experiments above suggested that interaction with GEF proteins were not sufficient to localise Rab31 to the membrane, we asked whether GEF proteins were at all necessary for Rab31's localisation. Two GEFs have been thus far identified for Rab31 – GAPex5 and RIN3. GAPex5 was first confirmed as a Rab31 GEF in studies looking at Glut4 trafficking. RIN3 was identified more recently as another Rab31 GEF. It has higher GEF activity for Rab31 than for the other Rab5 subfamily proteins such as Rab5 and Rab22 (Kajiho et al., 2011). A specific role for RIN3 has not yet been found.

We found that depletion of GAPex5 resulted in a dispersal of Rab31 from the TGN, as seen by a delocalisation with the trans-Golgi marker TGN46 (Fig. 3.3). This dispersal from the TGN did not result in an accumulation in other vesicular compartments, but instead suggested that the membrane association of Rab31 was affected. Interestingly, the same phenomenon was not observed when RIN3 was silenced (Fig. 3.3), as Rab31 remained largely at the TGN. Taken together, the results suggest that GAPex5 contributed to the TGN localisation of Rab31, and this was not due solely to its action as a GEF to activate Rab31, since RIN3 deficiency did not have the same effect.

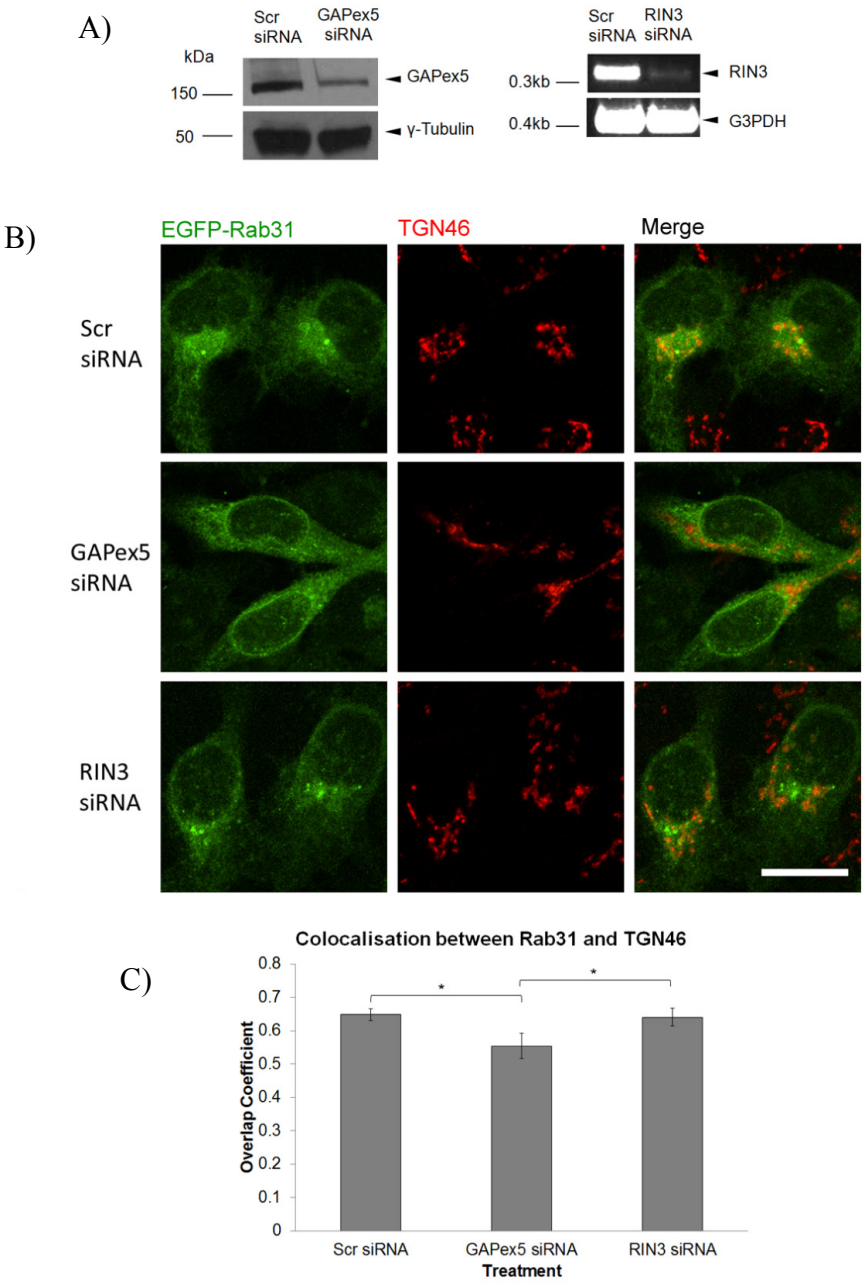


Fig. 3.3. Depletion of GAPex5 but not RIN3 disrupts Rab31 localisation to the TGN
A) A431 cells stably expressing EGFP-tagged Rab31 were transfected with Scrambled (Scr), GAPex5, or RIN3 siRNA, and assayed after 48 h. Cell lysates were analysed by Western blot for GAPex5 knockdown and RT-PCR for RIN3 knockdown. Band density of RIN3 was 90% reduced and GAPex5 was 70% reduced upon siRNA-mediated knockdown.
B) Cells were fixed and immunostained for localisation of EGFP-Rab31 (green) and TGN46 (red). Scale bar = 20 µm.
C) Colocalisation between EGFP-Rab31 and TGN46 was quantified using Zen 2010 software for calculation of the Overlap Coefficient. 27 cells in 3 independent experiments were analysed and data is shown as mean ± SEM. *P<0.05 as determined by one-way ANOVA.

In our survey on the effect of GAPex5 depletion on Rab31, we have used various antibody markers to label the different endosomal compartments, including the early endosomal marker EEA1. Interestingly, we observed that while Rab31 was largely TGN localised, there were a few Rab31 puncta colocalising with EEA1, suggesting that a percentage of Rab31 was also found in early endosomes. This is in line with the fact that EEA1 has been identified as an effector protein for Rab31, and is important for its role in EGFR trafficking (see Chapter 5). When GAPex5 was silenced, this colocalisation was also lost (Fig. 3.4).

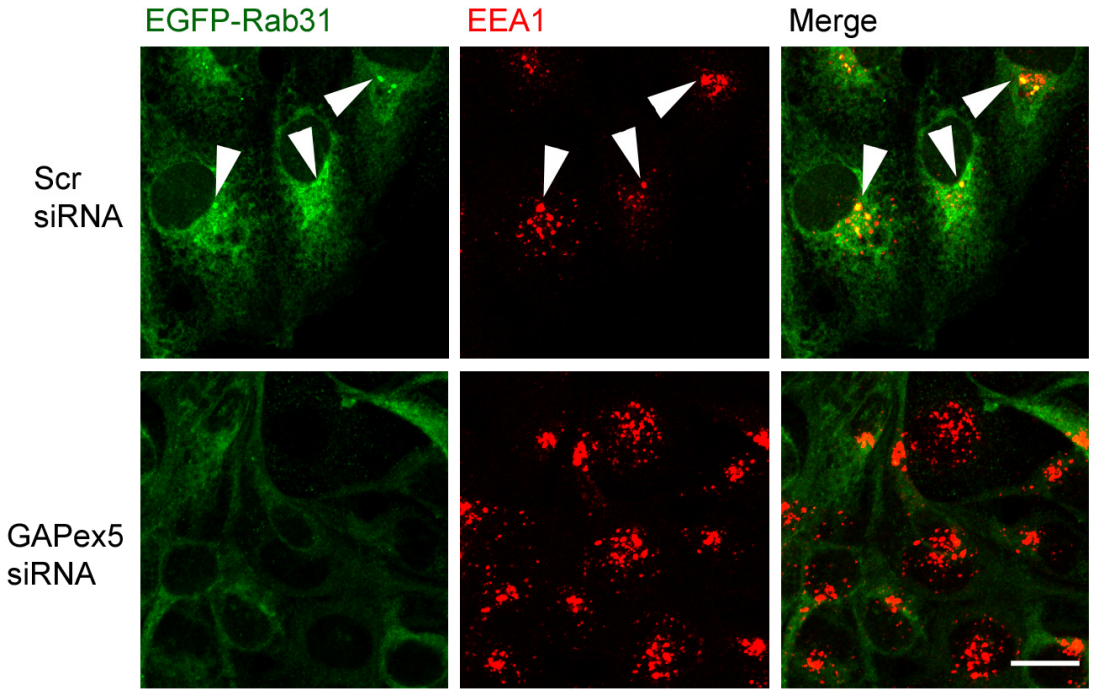


Fig. 3.4. Rab31 and its early endosomal localisation is lost when GAPex5 is silenced
A431 cells stably expressing EGFP-Rab31 were transfected with Scrambled (Scr) or GAPex5 siRNA and assayed after 48 h. Cells were fixed and immunostained for localisation of EGFP-Rab31 (green) and EEA1 (red). Arrowheads indicate some of the puncta that are positive for both Rab31 and EEA1. Scale bar = 20 μm.

Because of the different effects of GAPex5 and RIN3 deficiency on Rab31's membrane association, we explored the localisation of these GEFs themselves. We hypothesised that GAPex5 might be localised at the TGN, therefore enabling Rab31 to be localised there, whereas RIN3 might not. However, we found that GAPex5 staining was largely cytosolic (Fig. 3.5), when expressed in A431 cells. This is in line with observations by other groups, which found that endogenous GAPex5 in HeLa cells is cytosolic as well as on plasma membrane and endosomes (Hunker et al., 2006). As such, how GAPex5 can play a role in the membrane localisation of Rab31 remains to be determined. Unexpectedly, we found that RIN3 was localised to the TGN (Fig. 3.6). This is in contrast to previous studies which suggested that RIN3 was largely cytosolic (Kajiho et al., 2011). The difference in observation may be due to the fact that in the study by Kajiho et al., RIN3 was overexpressed, whereas in A431 cells the endogenous RIN3 levels were detectable by our RIN3 antibody.

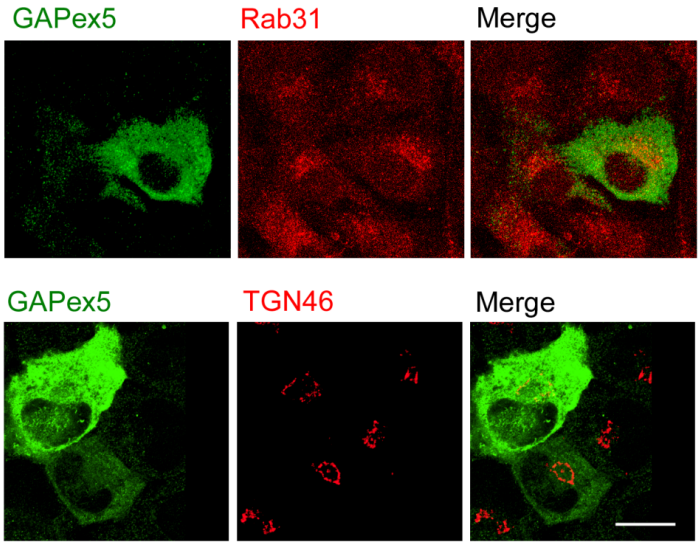


Fig. 3.5.GAPex5 is cytosolic and does not colocalise with Rab31 or TGN46
A431 cells were transfected with GAPex5 and immunoassayed after 48 h for GAPex5 (green) and Rab31 or TGN46 (red). Scale bar = 20 μm.

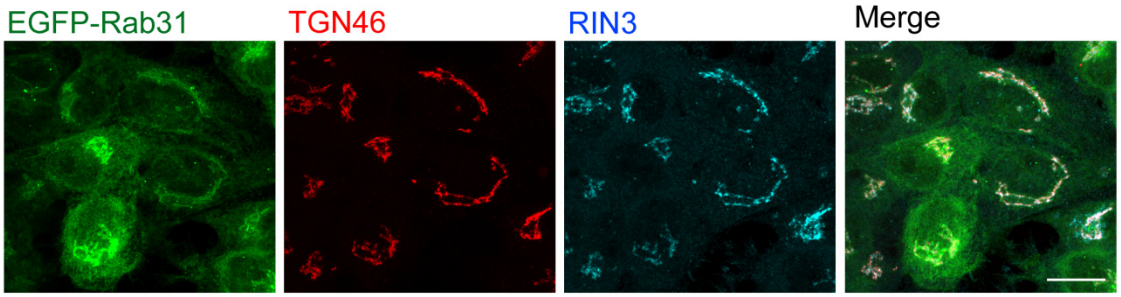


Fig. 3.6. Rab31 and RIN3 localise to the TGN
EGFP-Rab31 (green) was stably expressed in A431 cells. Cells were fixed and immunostained for TGN46 (red) and RIN3 (pseudo-coloured blue). Scale bar = 20 μm.

Dependence of Rab31 membrane localisation on effectors

Lastly, we checked if the depletion of a Rab31 effector protein, Early endosome antigen 1 (EEA1), would affect its subcellular localisation. We found that depletion of EEA1 did not affect the TGN localisation of Rab31 (Fig. 3.7). Because of the already low levels of colocalisation between Rab31 and EEA1, and the lack of a stable early endosome marker besides EEA1, we were unable to determine if EEA1 depletion also reduced the presence of Rab31 on the early endosome.

Another recently identified effector for Rab31 is the Adaptor protein, phosphotyrosine binding (PTB) domain, pleckstrin homology (PH) domain, leucine zipper-containing protein 2 (APPL2). APPL2 is found on the plasma membrane and in the cytosol (Wang et al., 2009). Its depletion also did not affect Rab31 localisation (Fig. 3.8).

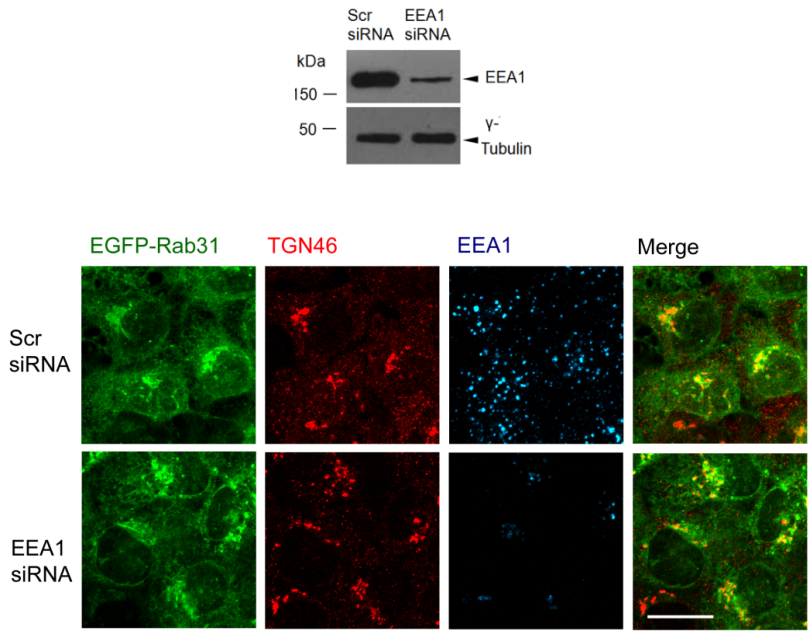


Fig. 3.7. Depletion of EEA1 does not disrupt localisation of Rab31
EGFP-Rab31 (green) was stably expressed in A431 cells. Cells were treated with scrambled (Scr) or EEA1 siRNA and assayed after 48 h. EEA1 was depleted by 80% as quantified by Western blot. Cells were fixed and immunostained for TGN46 (red) and EEA1 (pseudo-coloured blue). Merged panels show EGFP-Rab31 and TGN46. Scale bar = 20 μm.

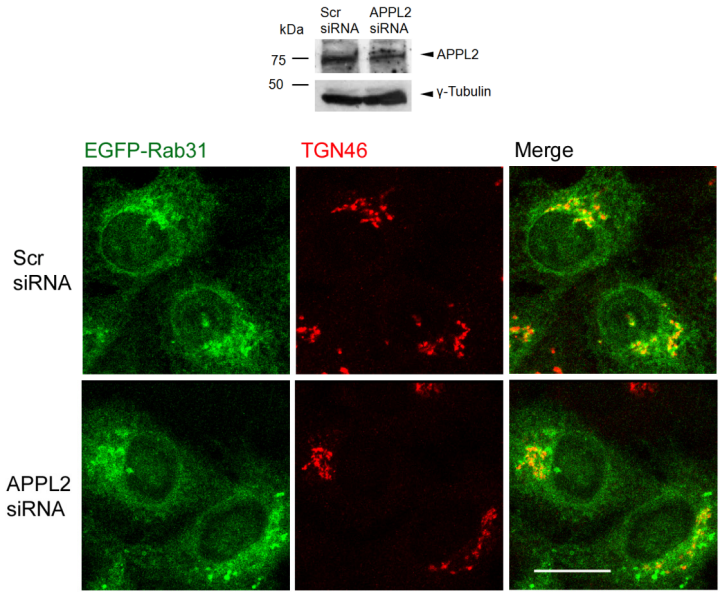


Fig. 3.8. Depletion of APPL2 does not disrupt localisation of Rab31
EGFP-Rab31 (green) was stably expressed in A431 cells. Cells were treated with scrambled (Scr) or APPL2 siRNA and assayed after 48 h. APPL2 was depleted by 50% as quantified by Western blot. Cells were fixed and immunostained for TGN46 (red). Scale bar = 20 μm.

3.4 Chapter Discussion: Factors influencing Rab31 subcellular localisation

We have shown that a) an intact HV domain, b) an intact C-terminal prenylation region, and c) the GEF GAPex5 are necessary for the localisation of Rab31 to the TGN/endosomal membrane. At the same time, however, any of the above alone are not sufficient to determine the correct membrane localisation of Rab31, as the GDP-locked Rab31 dominant negative mutant Rab31 S19N, which has an intact HV and GG domain and is able to engage GAPex5, is also not membrane localised. This might suggest that Rab31 must be able to go through the cycle of GDP-GTP exchange, and perhaps engage some as-yet-unknown cognate effector, to be correctly localised to the TGN membrane. While the effectors EEA1 and APPL2 do not appear to be necessary for Rab31 localisation, it must be noted that the knockdown in these studies were not complete (80% for EEA1 and 50% for APPL2), and further analysis may be required. It may also be that only one (as yet unidentified) 'key' effector is responsible, as suggested by the observations of Aivazian et al. (2006) where only the effector TIP47 but not the effector p40 is responsible for Rab9 localisation. As yet, there have not been other well-characterised interacting proteins of Rab31, especially those found on the TGN that could serve as anchors. As further work reveals more candidate Rab31-interacting proteins, we may be able to further explore this. Amongst the Rab31-interacting proteins that remain to be identified would also include putative Rab31 GAPs. At present, only two Rab31-interacting proteins, identified via a GST pulldown assay, have been shown to have the putative RabGAP TBC domain (Kanno et al., 2010). A direct role for these proteins serving as Rab31 GAPs has not been shown.

One interesting observation was that it was the deficiency of a specific GEF, GAPex5 but not RIN3, that affected Rab31 membrane localisation. Although the

silencing was incomplete (90% reduction for RIN3 and 70% for GAPex5), the results suggest that GAPex5 had a greater impact on Rab31 membrane localisation than RIN3. What would be the reason for the difference in effect between depletion of GAPex5 and RIN3? One reason could be that GAPex5 is the major GEF for cellular Rab31, whereas RIN3 may not have an extensive role. RT-PCR suggests that GAPex5 transcripts are more abundant than RIN3, in A431 cells at least (Fig. 3.9).

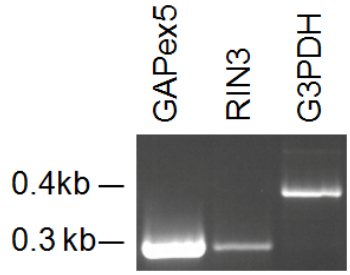


Fig. 3.9. RT-PCR to compare the endogenous levels of GAPex5 and RIN3 in A431 cells
Total mRNA was harvested from A431 cells and RT-PCR was performed using specific GAPEx5, RIN3 and G3PDH primers. After 35 cycles, amplified cDNA products were resolved on a 1.5% agarose gel and the bands visualised by ethidium bromide staining. Transcript of the housekeeping gene G3PDH is also amplified as a control.

Depletion of RIN3 therefore may not have as severe an effect as depletion of GAPex5 in terms of membrane targeting of Rab31. Also, there has been previous evidence to suggest that the various GEFs do not have an equal importance in facilitating nucleotide exchange of their target Rabs. For example, Rabex5, GAPex5 and RIN1 are all known GEFs of Rab5, but have varying efficiencies in terms of Rab5 GDP-GTP exchange (Kajiho et al., 2011). Another reason could be that different GEFs serve different roles in the cell. For example, RIN1 has been shown to be the major GEF important for the role of Rab5 in EGFR trafficking (Chen et al., 2009), whereas Rabex5 has been implicated in other functions of Rab5 such as stabilising the Rab5 microdomain (Lippé et al., 2001). As GAPex5 and RIN3 have different steady state

localisations in A431 cells, they likely have different principle roles. GAPex5, as the predominantly cytosolic GEF, may be in a better position to activate newly synthesised cytosolic Rab31. Once in its active state, Rab31 may then be able to interact with various other effector proteins at the TGN and form a microdomain. A third reason could be that the activity of GEFs themselves could be regulated by other events such as activation of signalling proteins triggered by external stimuli. For example, RIN3 could have regulatory domains that suppress GEF activity until stimulated; RIN3 may thus act as a GEF only in response to specific events. An example of this can be found in the Rab5 GEF Rabex5, in which the coiled-coil domains act as an autoinhibitory element that is relieved only upon binding to Rabaptin5 (Delprato and Lambright, 2007).

Taken together, the results do suggest that the presence of Rab31 on the TGN at steady state is a multifactorial process. Rab31 S19N, because it cannot exchange GDP for GTP, is perhaps unable to engage the effector proteins that would localise it to the TGN. Meanwhile, disruption of the HV and/or prenylation region may affect the binding of Rab31 to GAPex5. For this reason, our Rab31 truncation mutants are therefore not colocalised to the TGN. In our hands, we have been unable to isolate Rab31 and GAPex5 as a complex by co-immunoprecipitation, perhaps because of the transient nature of the interaction, or the inadequacies of the antibodies to co-immunoprecipitate. We were thus unable to verify if the ΔHV and ΔGG mutants were able to engage GAPex5.

Finally, we note the interesting observation of the presence of a small amount of Rab31 on the EEA1-containing endosomes. This may simply be part of a cycling of TGN-residing proteins or perhaps triggered by an external signalling event. It is interesting to note that the effectors of Rab31 identified thus far exist on endosomes,

and may suggest that Rab31 also has a role in endosomes. Results presented in the following chapters will explore this further.

4. Role of Rab31 in EGFR trafficking

4.1 Chapter Introduction: Rab proteins in trafficking of cell surface receptor EGFR

As described in Section 1.2.2., many Rabs, including those of the Rab5 subfamily, have been implicated to varying extents in various steps of the EGFR trafficking pathway. For example, Rab5 (Huang et al., 2004) and Rab 21 (Simpson et al., 2004) have been shown to enhance the movement of EGFR from the cell surface into early endosomes, while Rab22, and also Rab21, have been implicated in the later trafficking steps, with a general role in recycling or terminating the EGFR signalling in late endosomes / lysosomes (Kauppi et al., 2002). In some studies, loss of Rab5 activity (either by siRNA or use of dominant negative Rab5) has also bee n shown to inhibit the exit of ligand-bound EGFR from the early endosome (Chen et al., 2009; Dinneen and Ceresa, 2004). Meanwhile, another Rab, Rab7, has been linked to the maturation of late endosomes carrying EGFR to lysosomes (Vanlandingham and Ceresa, 2009).

Rab31 has been shown to function in Golgi-endosome trafficking of mannose 6-phosphate receptor (Rodriguez-Gabin et al., 2001; Rodriguez-Gabin et al., 2009), but has not been directly implicated in endocytic trafficking pathways. As shown in Chapter 3, Rab31 is localised to the perinuclear region of the cell, colocalising with trans-Golgi network (TGN) markers such as TGN46 (Ng et al., 2007). The TGN compartment is a major focal point for many vesicular transport pathways in the cell (Gu et al., 2001). Given its subcellular location at the TGN, Rab31 is therefore situated at the crossroads of many trafficking steps. It also shares similar GEFs and effectors with other Rab5 subfamily members, notably GAPex5 and the early endosome antigen 1 (EEA1) (Lodhi et al., 2007; Mishra et al., 2010). GAPex5 has been identified to bind to

EGFR through Cbl, an E3 ubiquitin ligase, upon receptor dimerisation (Su et al., 2007) while EEA1 participates in endosomal tethering and docking (Mills et al., 2001). As discussed in Section 3.4, we also found Rab31 localised to early endosomes. Given these connections listed above, we investigated if and how Rab31 would affect the EGFR trafficking itinerary in cultured A431 cells, a human epidermoid carcinoma line that has a high level of EGFR expression.

4.2 Results: Rab31 in endocytosis and degradation of EGFR

Rab31 affects the endocytic trafficking of ligand-bound EGFR

By depleting Rab31 in A431 cells, we have previously shown that loss of Rab31 inhibits the endocytic traffic of ligand-stimulated EGFR. Rab31 depletion delayed the entry of ligand-bound EGFR into late endosomes, as seen by the reduced fraction of large EGFR-positive puncta at 60 min post-pulse, and quantified by colocalisation with the late endosome marker CD63/Lamp3 (Ng et al., 2009). In investigating this phenomenon further, we first ascertained that depletion of Rab31 did not affect the plasma membrane levels of EGFR, suggesting that we are not observing a defect in anterograde transport of EGFR (Fig. 4.1).

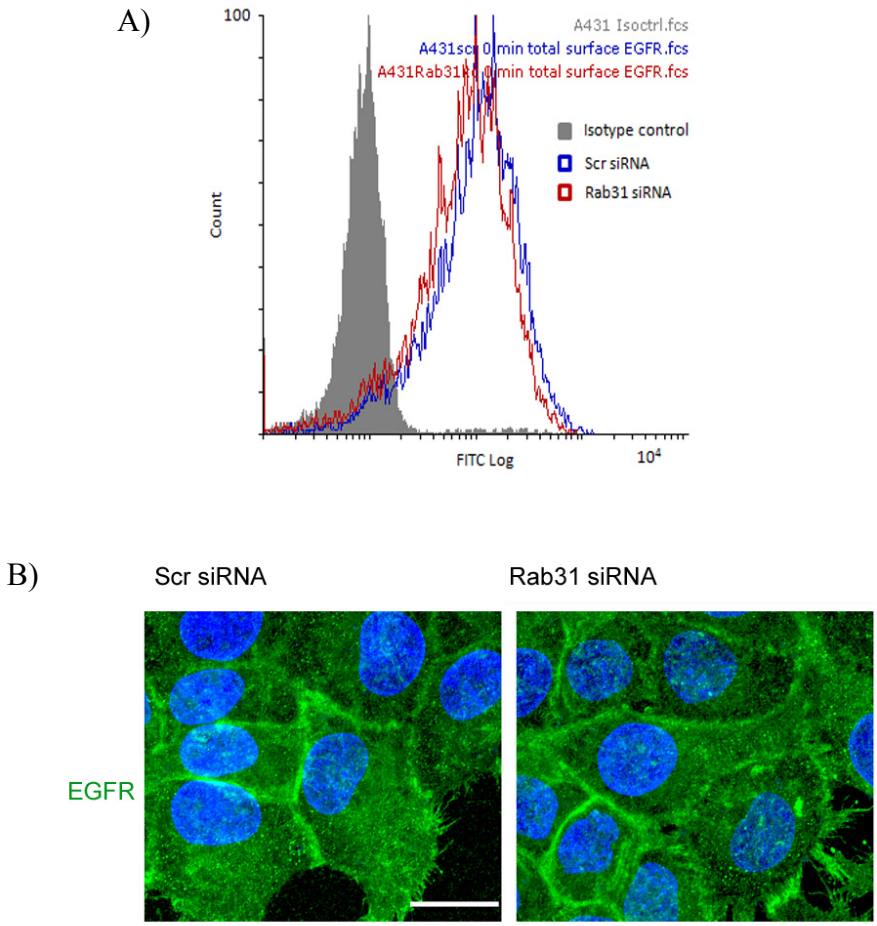


Fig. 4.1. Depletion of Rab31 does not affect plasma membrane levels of EGFR
A) Flow cytometry was used to analyse surface EGFR levels of non-permeabilised A431 cells, transfected with Scrambled siRNA (Scr) or Rab31 siRNA, and probed with FITC-labelled anti-EGFR antibodies. Histogram displaying count of fluorescent intensity is given.
B) A431 cells treated with Scr or Rab31 siRNA were immunostained for EGFR (green). No difference in EGFR levels at the plasma membrane was observed. Scale bar = 20 μm.

We next found that loss of Rab31 did not affect the colocalisation between ligand-bound EGFR (as indicated by labelled EGF signal) and EEA1 at 10 min post-pulse, suggesting that Rab31 is not required for the initial endocytosis step and trafficking of ligand-bound EGFR into the early endosome (Fig. 4.2), a role that is commonly attributed to Rab5 (Barbieri et al., 2000). Instead, loss of Rab31 resulted in a significantly diminished entry of ligand-bound EGFR into the late endosome compartment, as there is less colocalisation between ligand-bound EGFR and the late endosome marker CD63 at 30 min (Fig. 4.3). This time of 30 min corresponds to the transition of the internalised EGFR between early and late endosomes (Liu et al., 2009), and suggests that Rab31 is required for early endosome to late endosome transport of ligand-bound EGFR. To ascertain that the effect of Rab31 manipulations on endocytic traffic of ligand-bound EGFR was not specific only to A431 cells, which have exceptionally high levels of EGFR, we also performed similar experiments in HeLa cells. As with A431 cells, Rab31 depletion in HeLa cells also inhibited late endosome traffic of ligand-bound EGFR (Fig. 4.4).

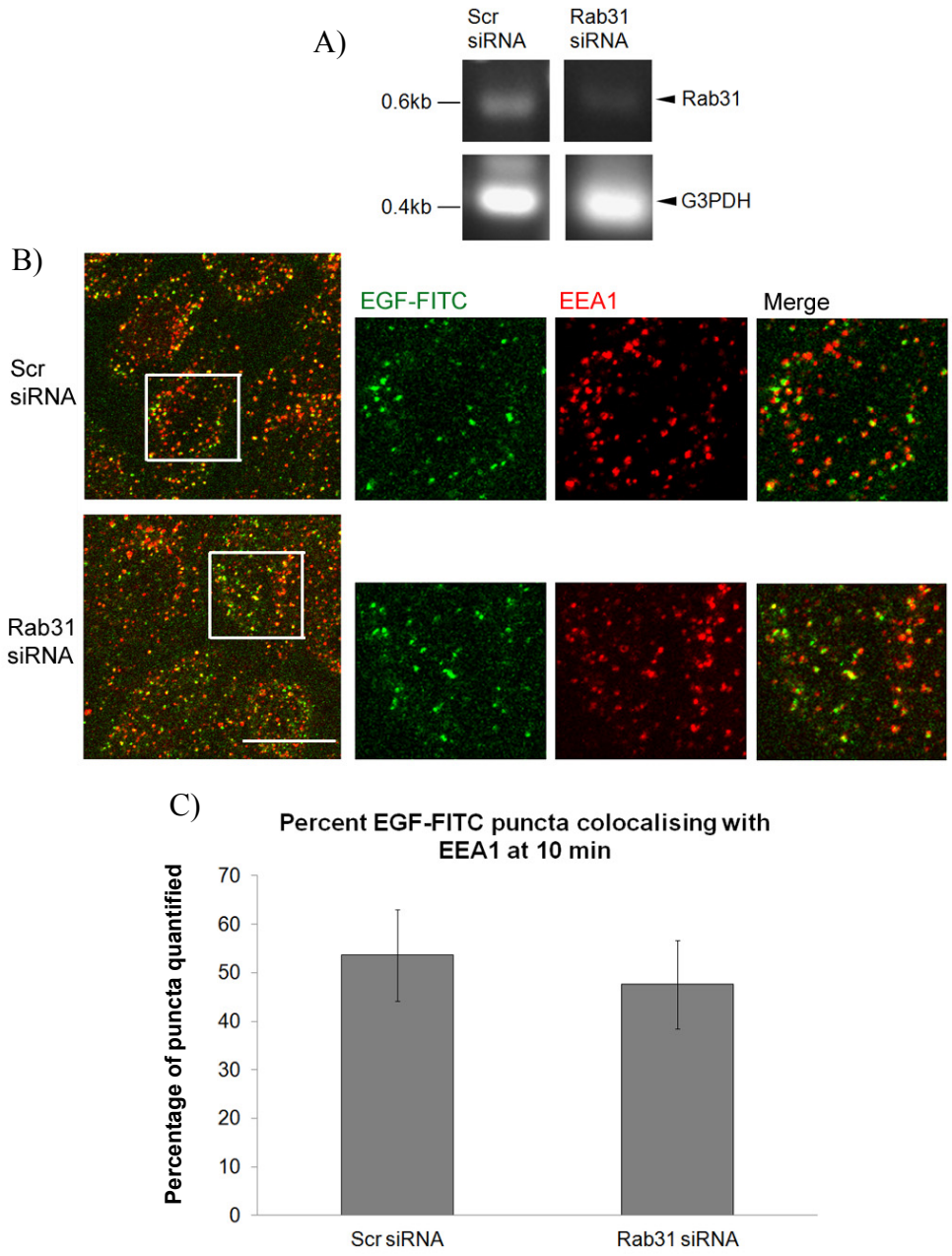


Fig. 4.2. Loss of Rab31 does not hinder early endocytic trafficking of ligand-bound EGFR

A431 cells were transfected with Scrambled (Scr) or Rab31 siRNA, and analysed for EGF trafficking after 48 h.

A) Rab31 was depleted by 85% as assessed by RT-PCR (the endogenous levels of Rab31 protein is low and Western blot analysis was not useful).

B) Cells were pulsed with 0.5 µg/mL EGF-FITC (green) followed by a 10 min chase before fixation and immunofluorescence analysis with the co-labelling of early endosome marker EEA1 (red). The individual and merged fluorescence signals of the areas enclosed in white squares are shown enlarged 2x on the right. Scale bar = 20 µm.

C) Number of EGF-FITC puncta positive for EEA1 was quantified and presented graphically as a percentage of total EGF-FITC puncta counted. 27 cells in 3 independent experiments were analysed and data is shown as mean ± SEM.

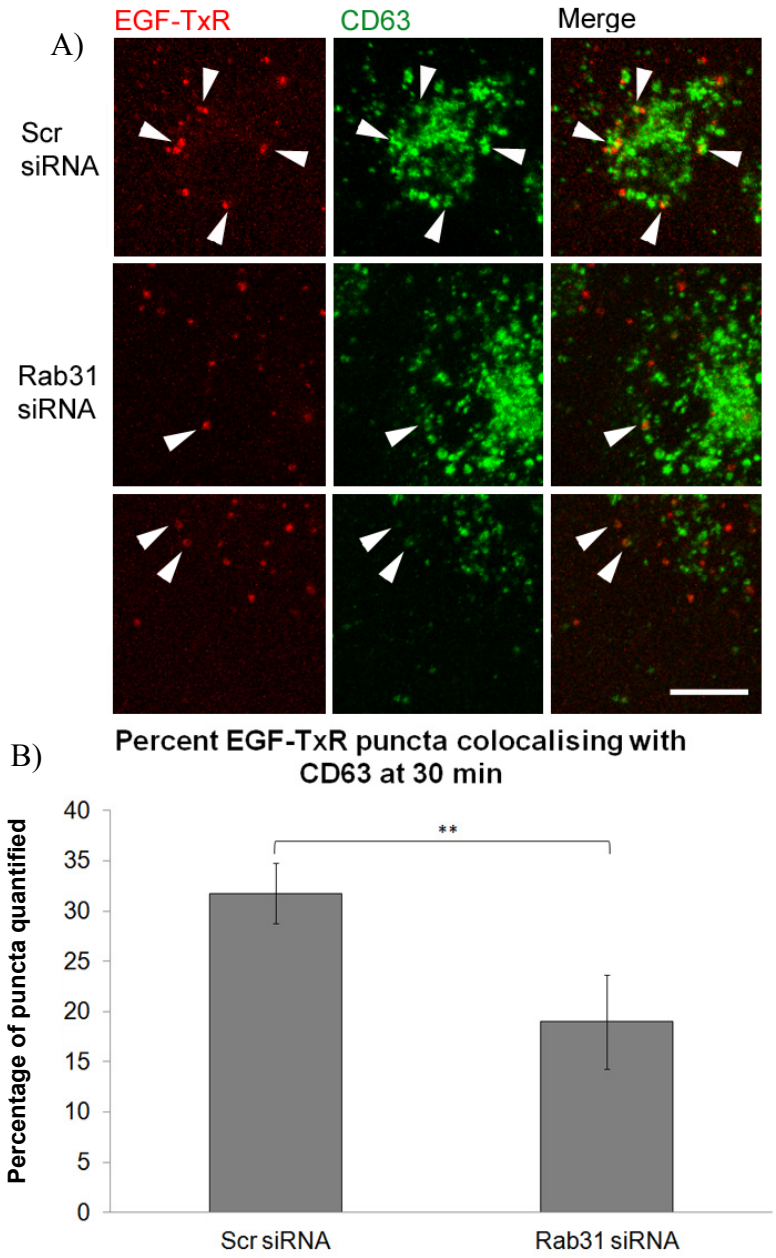


Fig. 4.3. Loss of Rab31 hinders trafficking of ligand-bound EGFR to the late endosome

A431 cells were transfected with Scrambled (Scr) or Rab31 siRNA, and analysed for EGF trafficking after 48 h. Cells were pulsed with 0.5 µg/mL EGF-TxR (red) followed by a 30 min chase before fixation and immunofluorescence analysis with the co-labelling of the late endosome marker CD63 (green).

A) Arrowheads show EGF-TxR puncta colocalising with CD63. Scale bar = 20 µm.

B) EGF-TxR puncta also positive for CD63 was quantified and presented graphically as a percentage of total EGF-TxR puncta counted. 27 cells in 3 independent experiments were analysed and data is shown as mean ± SEM. **P<0.01 by Student's t-test.

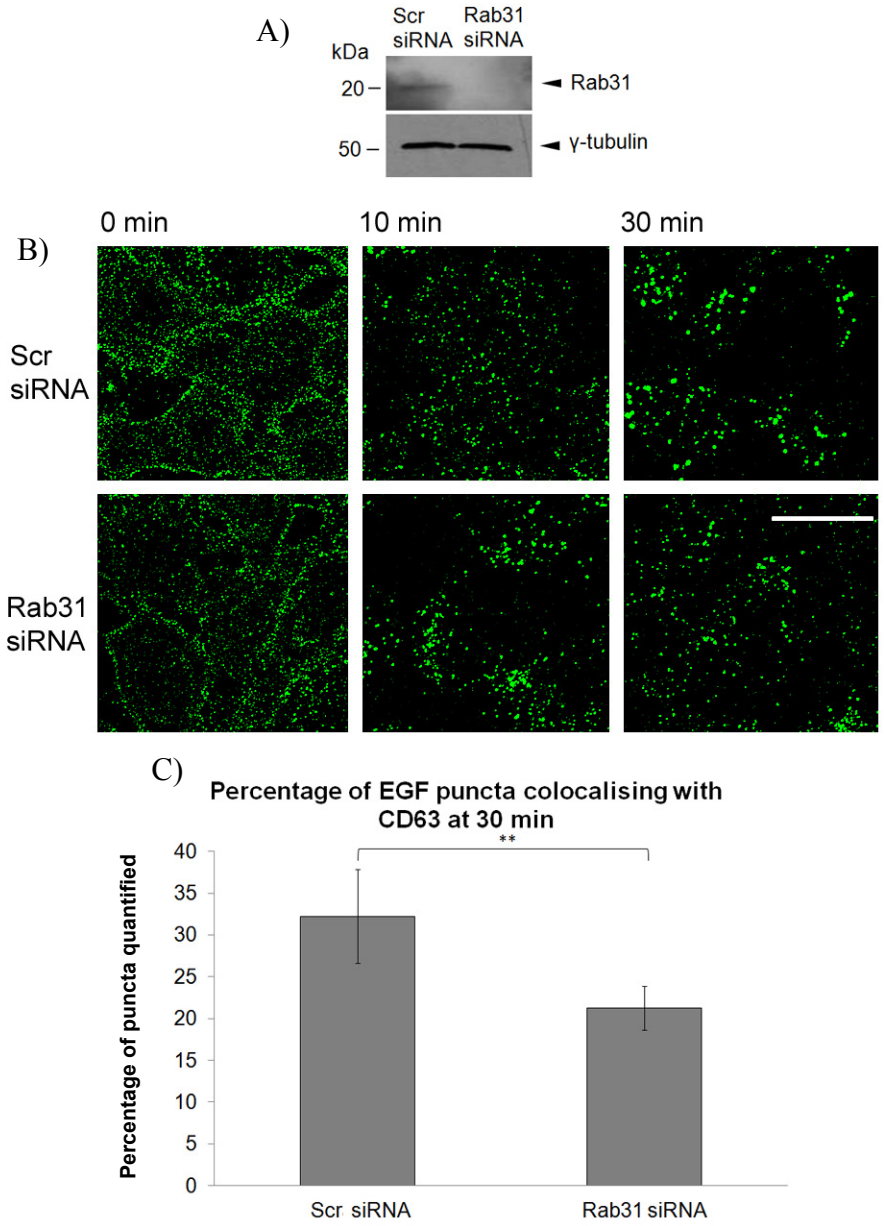


Fig. 4.4. Loss of Rab31 hinders trafficking of ligand-bound EGFR in HeLa cells to the late endosome

HeLa cells were transfected with Scrambled (Scr) or Rab31 siRNA and analysis was performed after 48 h.

A) Rab31 was depleted by 85% as quantified by Western blot.

B) Cells were pulsed with 0.5 µg/mL EGF-FITC (green) followed by a chase before fixation and immunofluorescence. At 30 min, larger EGF-FITC puncta is observed for cells treated with Scr compared to Rab31 siRNA. Scale bar = 20 µm.

C) Cells were pulsed with 0.5 µg/mL EGF-FITC followed by a 30 min chase before fixation and immunofluorescence analysis with the co-labelling of the late endosome marker CD63. Number of EGF-FITC puncta also positive for CD63 was quantified and presented graphically as a percentage of total EGF-FITC puncta counted. 35 cells in 3 independent experiments were analysed and data is shown as mean ± SEM. **P<0.01 by Student's t-test.

We asked if an increase in Rab31 levels could have a converse effect on the endocytic transport of EGFR. Stable overexpression of Rab31 (Rab31 OE) did not affect the steady state levels or plasma membrane localisation of EGFR when compared to cells transfected with the vector control (VC) (Fig. 4.5).

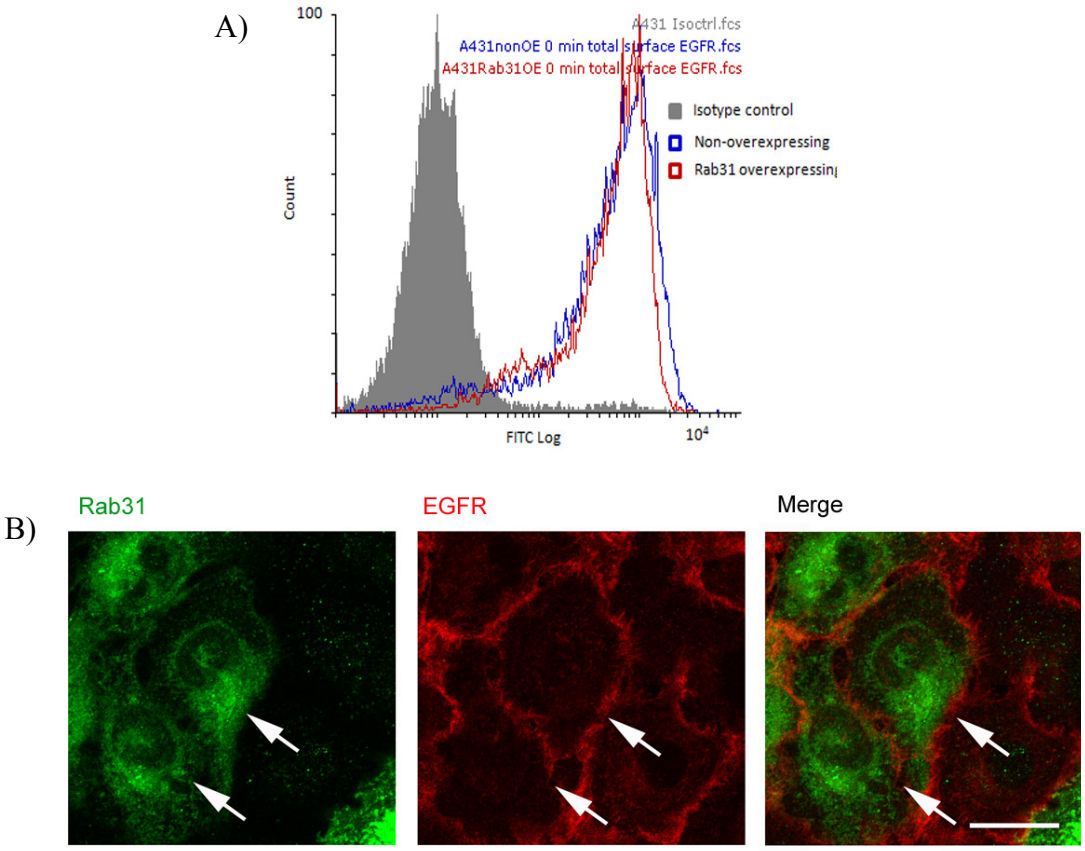


Fig. 4.5. Overexpression of Rab31 does not affect plasma membrane levels of EGFR
A) Flow cytometry was used to analyse surface EGFR levels of non-permeabilised A431 cells, overexpressing Rab31 or not, probed with FITC-labelled anti-EGFR antibodies. Histogram displaying count of fluorescent intensity is given.
B) Rab31 was stably overexpressed in A431 cells and immunostained for Rab31 (green) and EGFR (red). No difference in EGFR levels at the plasma membrane was observed between Rab31-overexpressing cells (arrows) and non-expressing cells. Scale bar = 20 µm.

When the cells were pulsed and chased with EGF-TxR, cells overexpressing Rab31 exhibited larger EGF-TxR punctate structures representative of internalised EGFR, as observed by immunofluorescence (Fig. 4.6A). The difference was only evident at 30 min, not at 10 min post-pulse, suggesting that Rab31 overexpression does not impact the initial internalisation of EGFR from the plasma membrane. There was significantly more large puncta (>0.25 μm) in the Rab31 OE cells, whereas there was significantly more small puncta (<0.05 μm) in the non-overexpressing cells from the same population (Fig. 4.6B). Moreover, overexpression of Rab31 increased the percentage of EGF-TxR puncta that were positive for CD63 at 30 min (Fig. 4.6C). These experiments were repeated in HeLa cells and similar results were obtained (Fig. 4.7). The results, beyond complementing the Rab31 depletion experiments, suggest that Rab31 positively influences the endocytic trafficking of ligand-bound EGFR, and that this influence may be proportionately enhanced by increasing Rab31 levels.

To confirm that Rab31 impacts the endocytic trafficking of ligand-bound EGFR to late endosomes, we looked at the eventual degradation of EGFR after an EGF pulse. HeLa cells were used as the changes in EGFR levels due to degradation were more easily seen in a Western blot due to the more moderate levels of EGFR in HeLa cells compared to A431 cells. We found that at 30 min, loss of Rab31 reduced the rate at which EGFR is degraded by 14%, while overexpression of Rab31 increased the rate by 17% (Fig. 4.8).

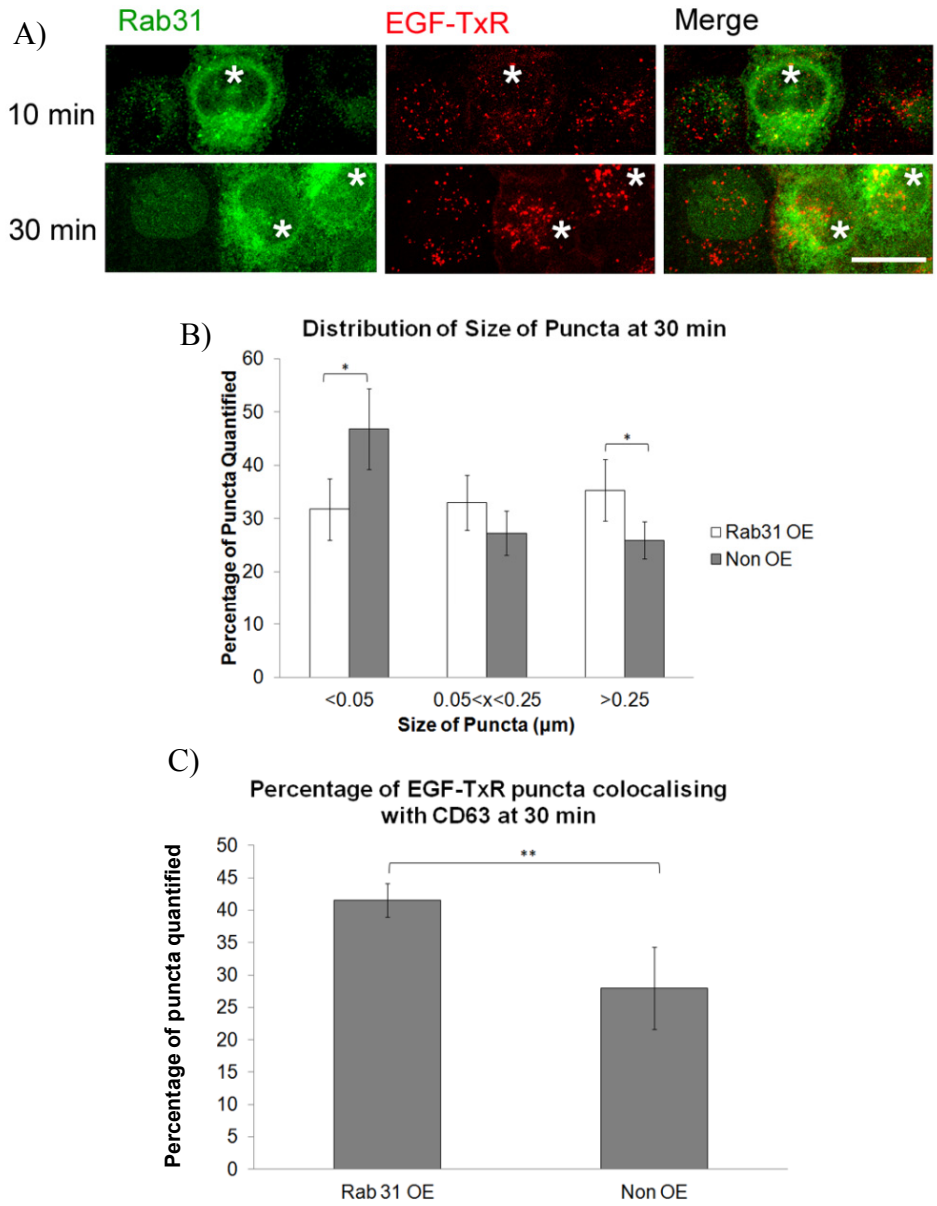


Fig. 4.6. Overexpression of Rab31 enhances endocytic trafficking of ligand-bound EGFR to the late endosome in A431 cells

A431 cells were stably transfected with Rab31. Cells were pulsed with 0.5 µg/mL EGF-TxR followed by a chase at various time points before fixation and immunofluorescence analysis.

A) Effect of overexpressing Rab31 (asterisks) on the endocytosis of EGF-TxR (red) was observed by labelling for Rab31 (green) after 10 and 30 min chases. Scale bar = 20 µm.

B) Sizes of EGF-TxR puncta in Rab31 overexpressing (OE) and non-OE cells from the same population were quantified using ImageJ, and the size distribution is represented graphically as a bar chart. 15 cells in 3 independent experiments were analysed and data is shown as mean ± SEM. *P<0.05 by Student's t-test.

C) Number of EGF-TxR puncta also positive for CD63 in Rab31 OE and non-OE cells from the same population was quantified and presented graphically as a percentage of total EGF-TxR puncta counted. 32 cells in 3 independent experiments were analysed and data is shown as mean ± SEM. **P<0.01 by Student's t-test.

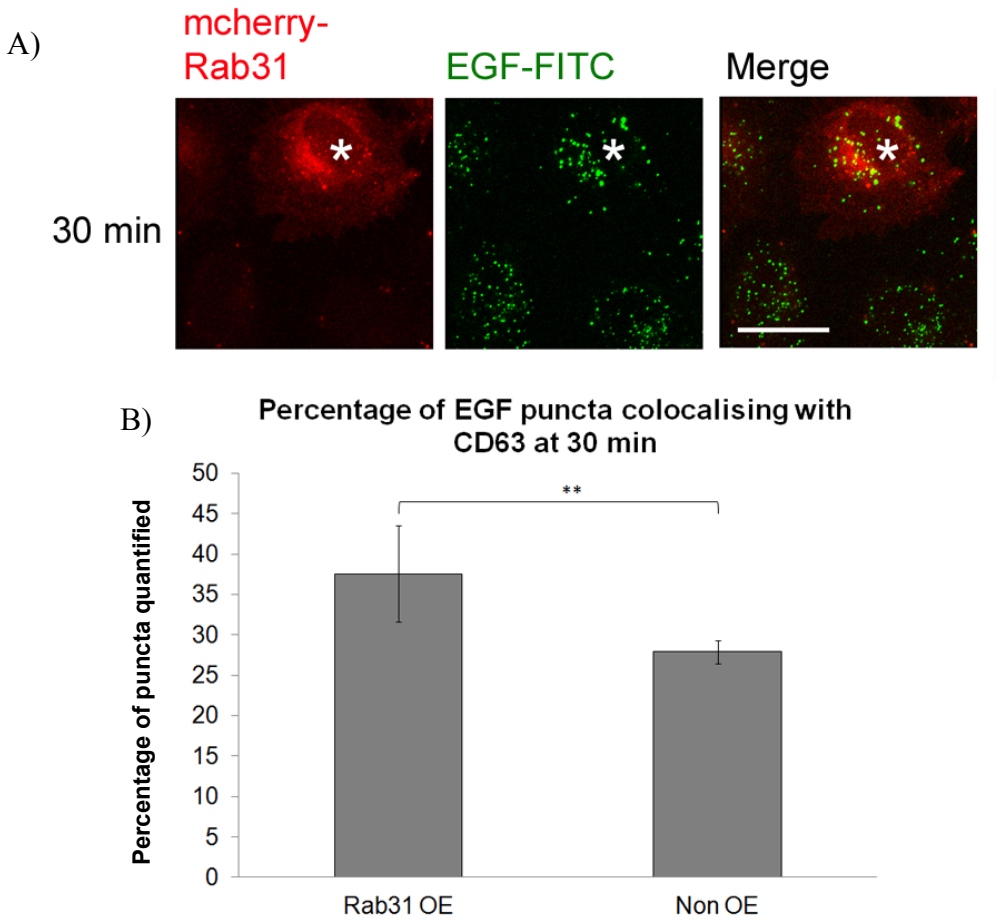


Fig. 4.7. Overexpression of Rab31 enhances endocytic trafficking of ligand-bound EGFR to late endosomes in HeLa cells

HeLa cells were stably transfected with mcherry-Rab31. Cells were pulsed with 0.5 µg/mL EGF-FITC followed by a chase at various time points before fixation and immunofluorescence analysis.

A) Effect of overexpressing mcherry-Rab31 (red) on the endocytosis of EGF-FITC (green) was observed after 30 min chase. There are larger puncta in the cell overexpressing Rab31 (asterisk) compared to the cells without overexpression. Scale bar = 20 µm.

B) Number of EGF-FITC puncta also positive for CD63 was quantified and presented graphically as a percentage of total EGF-TxR puncta counted. 35 cells in 3 independent experiments were analysed and data is shown as mean ± SEM. **P<0.01 by Student's t-test.

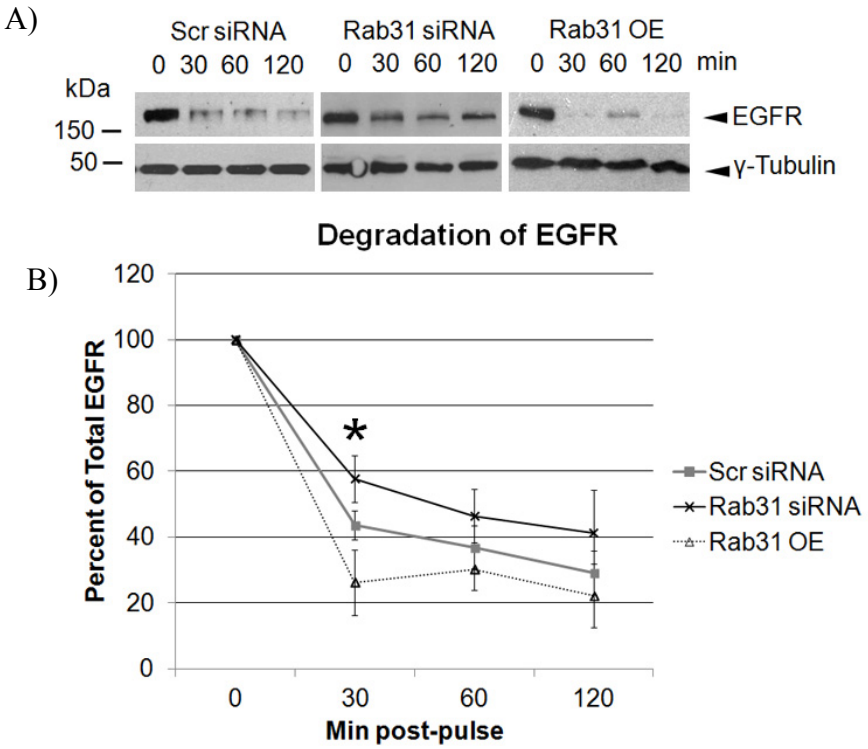


Fig. 4.8. Manipulation of Rab31 levels affects rate of degradation of ligand-bound EGFR

HeLa cells overexpressing Rab31 (Rab31 OE) and those that were transfected with Scrambled (Scr) or Rab31 siRNA were pulsed with 0.5 μg/mL EGF.

A) At the various time points indicated, lysates were harvested and probed by Western blot for total EGFR and γ-tubulin. Shown is a representative set of data from 6 independent experiments.

B) Levels of EGFR were normalised against γ-tubulin and plotted graphically as a percentage of EGFR at 0 min after pulse. Data represents mean ± SEM of 6 independent experiments. *P<0.05 between Scr and Rab31 siRNA, or Scr and Rab31 OE, at 30 min.

To verify that Rab31 is indeed the critical factor depleted in the depletion experiments, we attempted to rescue Rab31-silenced cells by transfecting the cells with Myc-tagged mouse Rab31 (Myc-mRab31), which differs in sequence from human Rab31 targeted by our siRNA. Overexpression of silencing-resistant Myc-mRab31 could indeed rescue the ligand-bound EGFR trafficking defect caused by loss of Rab31. After a 30 min chase with EGF-TxR, cells in which the rescue of Rab31 knockdown phenotype had occurred (as evidenced by the expression of Myc-tagged mRab31) displayed larger EGF-TxR puncta compared to cells in the same population that did not express Myc-mRab31 (Fig. 4.9A). Quantification of this phenomenon showed that there was significantly larger percentage of puncta that are >0.25 μm in cells overexpressing Myc-mRab31, compared to cells without overexpression or cells transfected with the vector control (Fig. 4.9B). We also quantified the percentage of EGF-TxR puncta that were positive for CD63 (arrowheads), after a 30 min chase (Fig. 4.10). In cells with Myc-mRab31 overexpression, the percentage of EGF-TxR and CD63 colocalisation was significantly increased compared to cells in the same population with no Myc-mRab31 expression. The percentage was also significantly higher compared to cells transfected with the empty vector. Taken together, our results suggest that Rab31 specifically plays a role in the endocytic trafficking of ligand-bound EGFR.

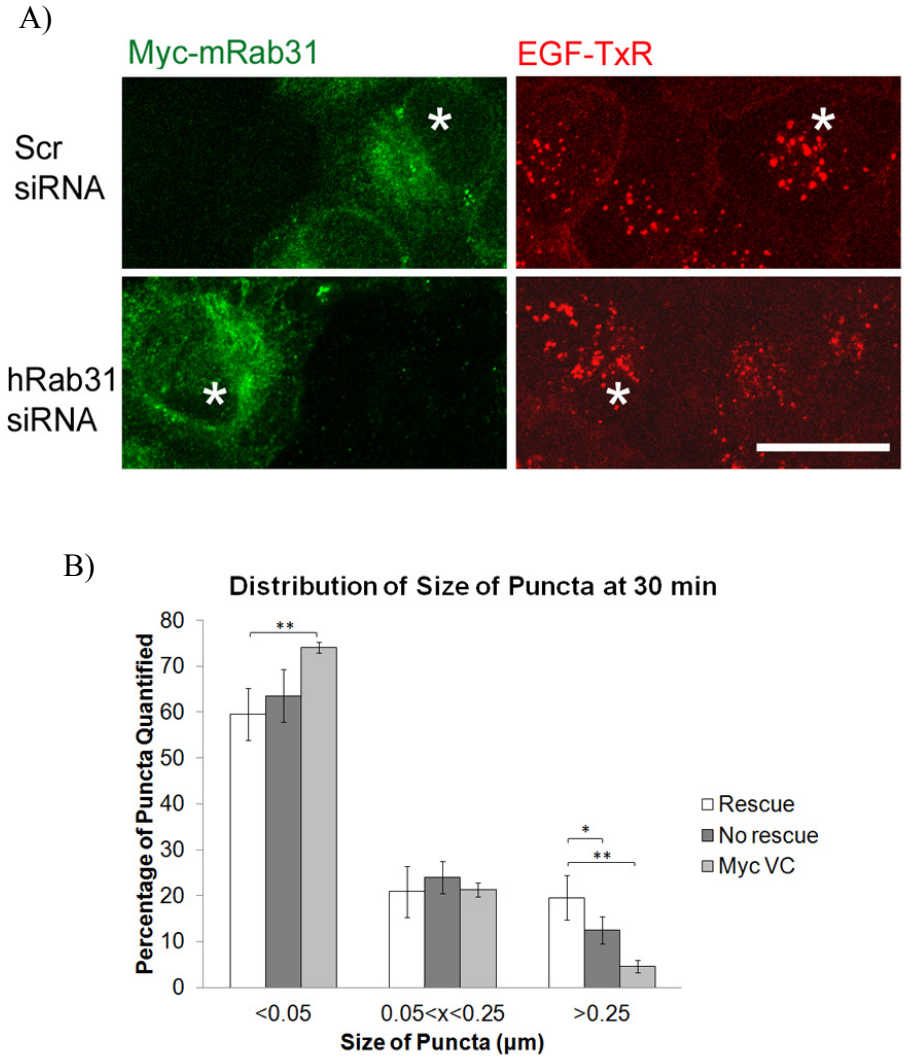


Fig. 4.9. Rescue of Rab31 depletion restores the endocytic trafficking defect of ligand-bound EGFR as quantified by puncta size

A431 cells were transfected with Scrambled (Scr) or Rab31 siRNA for 48 h before subsequent transfection with siRNA silencing-resistant Myc-tagged mouse Rab31 (Myc-mRab31) or empty Myc vector (Myc VC). Cells were pulsed 24 h later with 0.5 µg/mL EGF-TxR followed by chase and fixation at various time points.

A) Immunofluorescence analysis for EGF-TxR (red) and Myc-mRab31 (green). Asterisks indicate Myc-mRab31 overexpressing cells with larger EGF-TxR puncta compared to non-overexpressing cells. Scale bar = 20 µm.

B) Sizes of EGF-TxR puncta in cells with (Rescue), and without Myc-mRab31 expression (No rescue), and cells transfected with empty Myc vector (Myc VC) after Rab31 siRNA treatment were quantified using ImageJ, and the size distribution is represented graphically as a bar chart. 15 cells in 3 independent experiments were analysed and data is shown as mean ± SEM. *P<0.05 and **P<0.01 by Student's t-test.

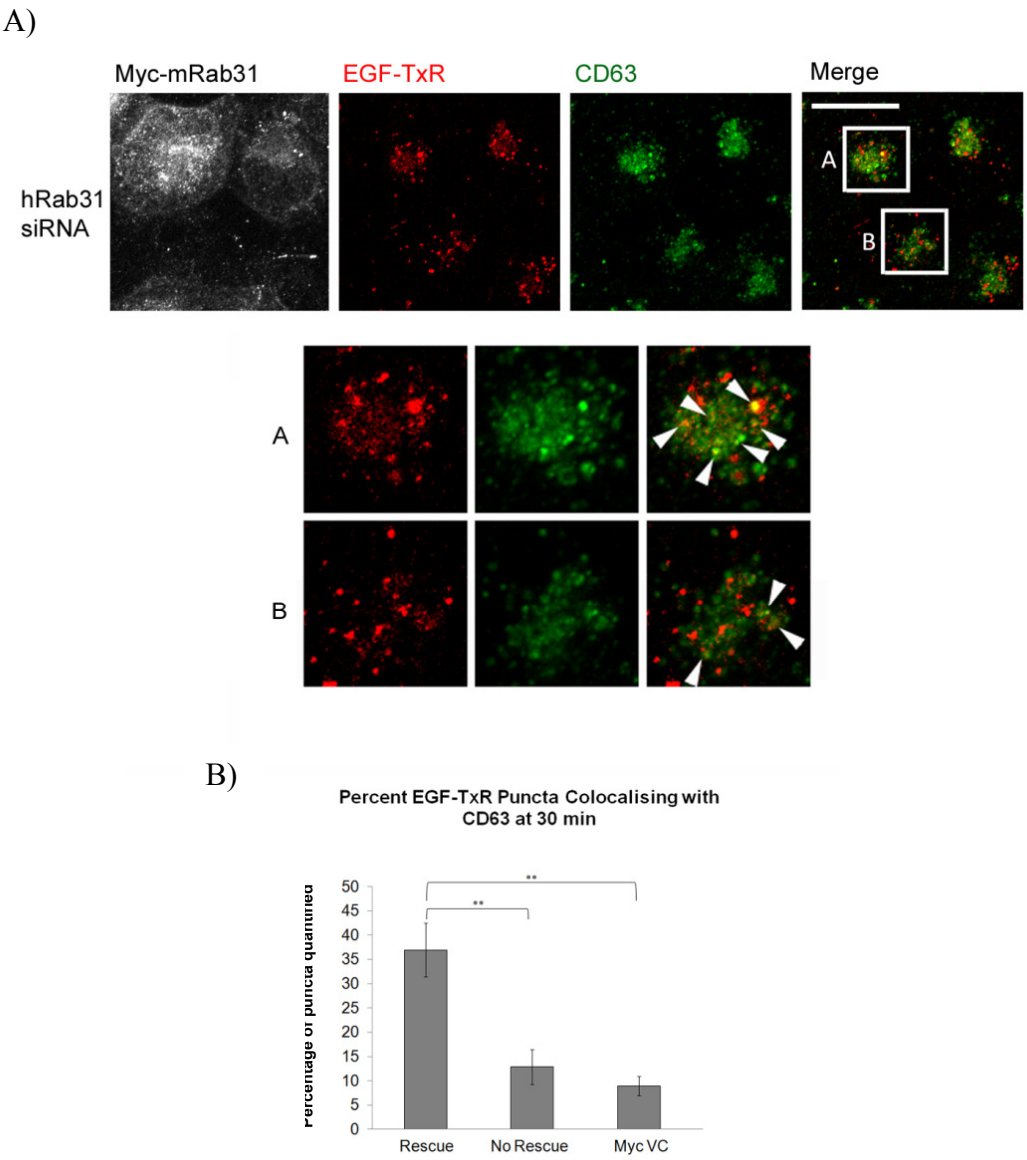


Fig. 4.10. Rescue of Rab31 depletion restores the endocytic trafficking defect of ligand-bound EGFR as quantified by colocalisation with CD63
A) The effect of depleting Rab31 and subsequent rescue was determined by assessing the amount of EGF-TxR (red) that have entered the CD63 (green)-containing late endosome. Box A encloses the central region of a representative cell with Myc-mRab31 expression (pseudo-coloured white) while Box B encloses a representative cell without. Lower panel shows individual and merged fluorescence signals of the boxed areas, magnified 2x. Arrowheads indicate some EGF-TxR puncta (red) that are also positive for CD63 (green). Scale bar = 20 µm.
B) The percentage of EGF-TxR puncta that are positive for CD63 in cells with (Rescue), or without Myc-mRab31 expression (No rescue) and cells transfected with empty Myc vector (Myc VC) after Rab31 siRNA treatment, were quantified and presented graphically as a percentage of total EGF-TxR puncta counted. 29 cells in 3 independent experiments were analysed and data is shown as mean ± SEM. **P<0.01 by Student's t-test.

Rab31 associates with an EGFR-trafficking complex

We sought to determine how Rab31 could influence the trafficking of EGFR. We found that Rab31 associated with EGFR in a GTP-dependent manner by co-immunoprecipitation with anti-Rab31 antibodies (Fig. 4.11A). Rab5, in comparison, co-immunoprecipitated lesser amounts EGFR. Similarly, we were able to affinity pulldown EGFR using glutathione S-transferase (GST)-Rab31, but not GST, in the presence of a non-hydrolysable GTP analogue GTPγS in both A431 and HeLa cells (Fig. 4.11B, C). In contrast, little or no EGFR was pulled down using GST-tagged Rab31 S19N, a dominant negative, GDP-locked mutant of Rab31 (Fig. 4.11D), suggesting that Rab31 must be in its active, GTP-bound form to associate with EGFR.

We looked closer at how Rab31 might be associated with EGFR during its endocytic traffic. Using immunofluorescence analysis of A431 cells stably transfected with EGFP-Rab31 and pulse-chased with EGF-TxR, we observed that a portion of the EGFP-Rab31 signal associates with the EGF-positive punctate structures indicative of endosomes bearing the ligand-bound EGFR. This association was particularly evident after a 30 min chase (Fig. 4.12A, B), a period when ligand-bound EGFR is likely to be transiting between early and late endosomes (Liu et al., 2009). We quantified the percentage of EGF-TxR puncta that are also immunopositive for Rab31, and found that the percentage increases upon EGF pulse and is statistically significant at 30 min. This suggests that a portion of Rab31 may become localised in the endosomal membrane rather than the TGN, and may associate as part of a trafficking complex with ligand-bound EGFR. We likewise observed a percentage of colocalisation between Rab31 and EGF in an orthogonal projection of HeLa cells transfected with mCherry-Rab31 and pulse-chased with EGF-FITC (Fig. 4.12C), suggesting that this phenomenon is not specific only to A431 cells. Time-lapse microscopy of A431 cells

stably expressing EGFP-Rab31 and pulsed with EGF-TxR also showed a gradual increase in colocalisation between Rab31 and EGF (Fig. 4.13).

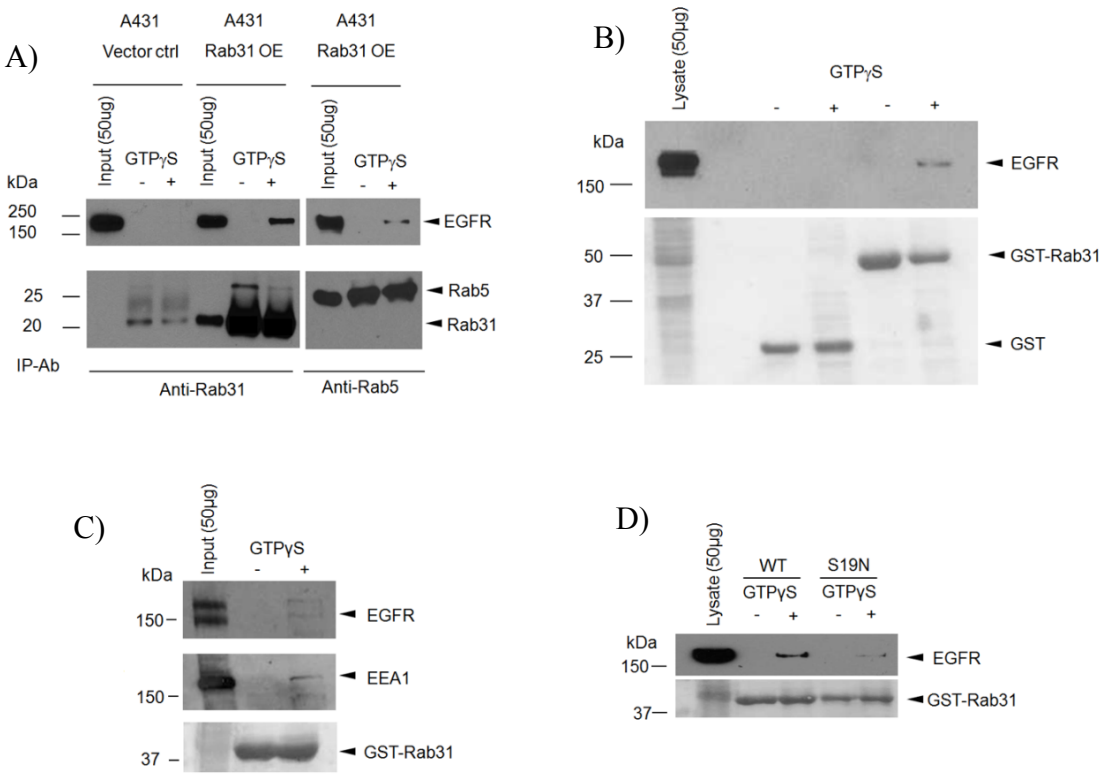
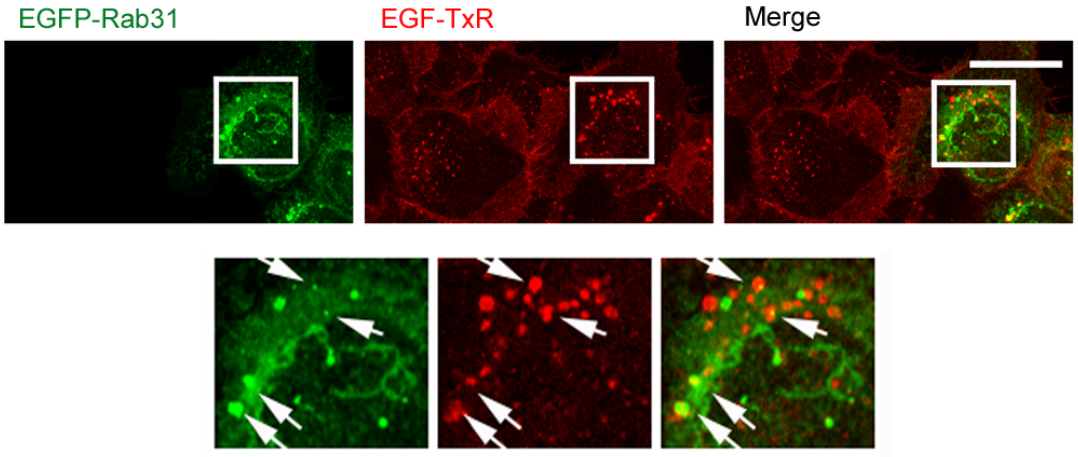
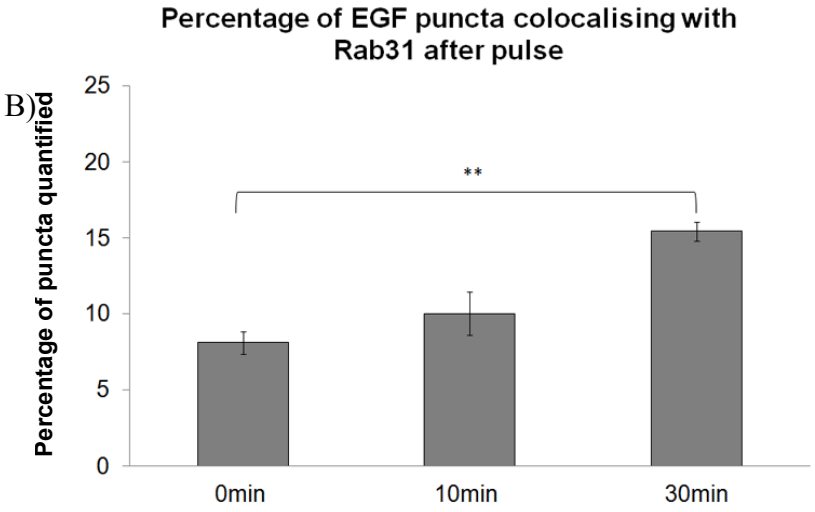


Fig. 4.11. Rab31 associates with EGFR by affinity assays
A) EGFR was co-immunoprecipitated with Rab31 or Rab5 antibody respectively, using 1 mg lysates from cells transfected with vector alone (Vector ctrl) and Rab31 (Rab31 OE) respectively, with or without GTPγS loading.
B) 1 mg of A431 cell lysate with and without GTPγS were incubated with 20 µg GST or GST-Rab31 and glutathione beads, and the ability of the GST fusion proteins to pulldown EGFR was analysed by Western blot. The GST fusion proteins were visualised with Ponceau S stain.
C) 1 mg of HeLa A431 cell lysate with and without GTPγS were incubated with 20 µg GST or GST-Rab31 and glutathione beads, and the ability of the GST fusion proteins to pulldown EGFR was analysed by Western blot. The GST fusion proteins were visualised with Ponceau S stain.
D) 1 mg of A431 cell lysate with and without GTPγS was incubated with 20 µg GST-Rab31 or GST-Rab31 S19N and glutathione beads, and the ability of the GST fusion proteins to pulldown EGFR was analysed by Western blot. The GST proteins were visualised with Ponceau S stain.

A)


EGFP-Rab31
EGF-TxR
Merge

B)


Percentage of EGF puncta colocalising with Rab31 after pulse
Percentage of puncta quantified
25
20
15
10
5
0
**
0min
10min
30min

C)

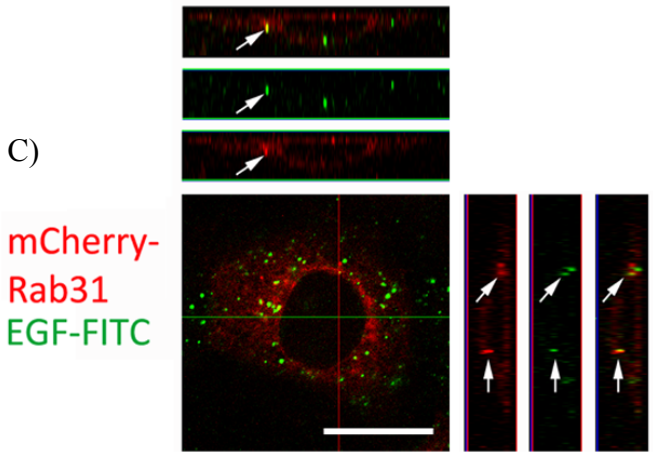

mCherry-Rab31
EGF-FITC

Fig. 4.12. Rab31 associates with EGFR 30 min after EGF pulse
A) A431 cells stably transfected with EGFP-Rab31 were pulsed with 0.5 μg/mL EGF-TxR, fixed after 30 min and analysed for colocalisation between EGFP-Rab31 (green) and EGF-TxR (red). The lower panel are the boxed areas in the upper panel, enlarged 2x. Arrows indicate structures positive for both EGFP-Rab31 and EGF-TxR. Scale bar = 20 μm.
B) Percentage of EGF-TxR positive puncta that are also positive for EGFP-Rab31 was quantified from cells fixed after 0, 10 and 30 min chase, and graphically represented as a percentage of total EGF-TxR puncta counted. 34 cells in 3 independent experiments were analysed and data is shown as mean ± SEM. **P<0.01 by Student's t-test.
C) HeLa cells were pulsed with 0.5 μg/mL EGF-FITC, fixed after 30 min and analysed for colocalisation between mcherry-Rab31 (red) and EGF-FITC (green) in an orthogonal projection of z-stacked images. Arrows indicate structures positive for mcherry-Rab31 and EGF-FITC. Scale bar = 20 μm.

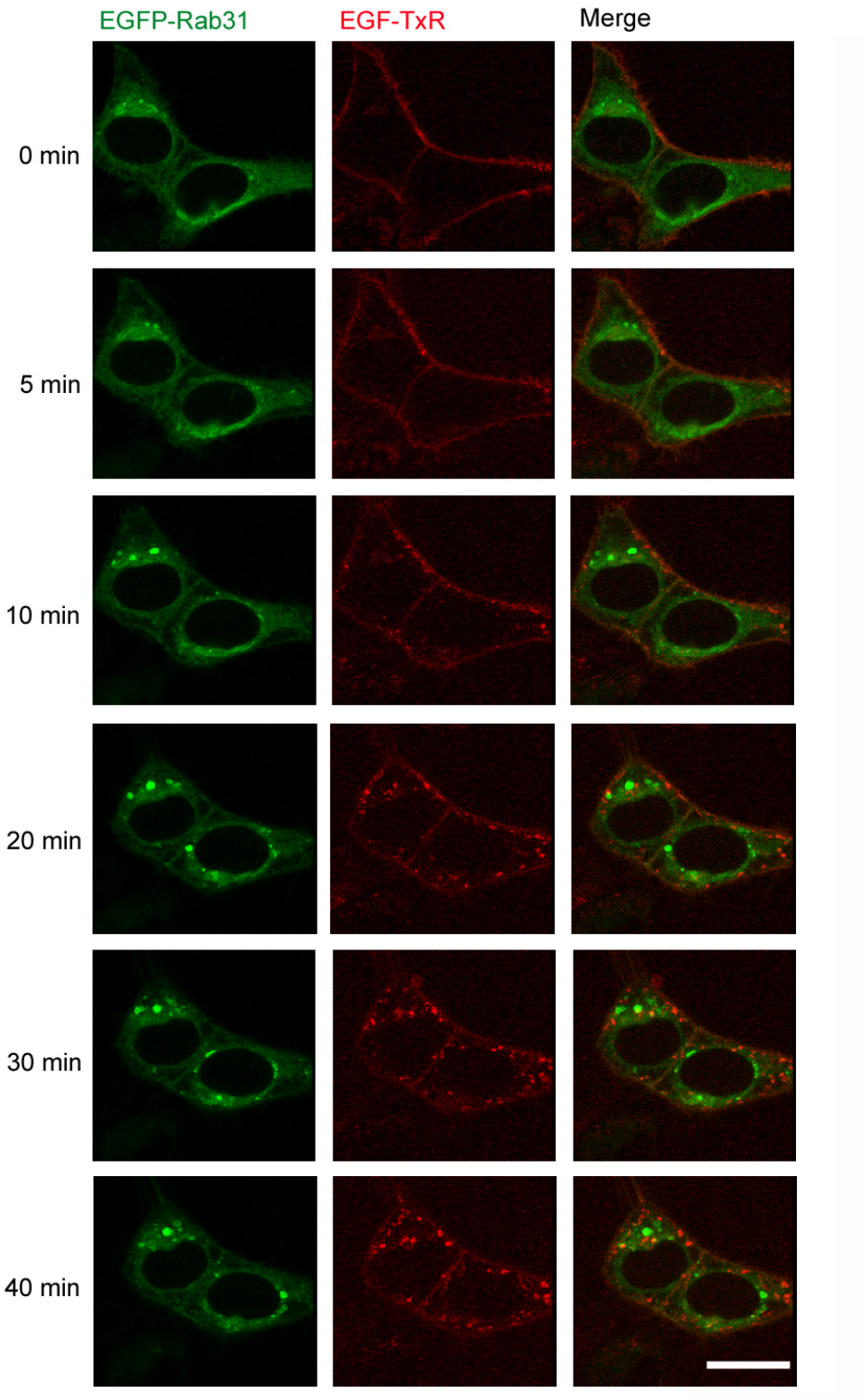


Fig. 4.13. Rab31 gradually increases in association with EGFR after EGF pulse as seen by live imaging
A431 cells stably transfected with EGFP-Rab31 were pulsed with 0.5 µg/mL EGF-TxR and imaged at various time points. There is partial colocalisation between EGFP-Rab31 puncta and EGF-TxR, gradually increasing from 10-40 min. Scale bar = 20 µm.

That Rab31 may indeed be part of an EGFR-containing trafficking complex was further supported by a glycerol gradient sedimentation analysis of proteins from Rab31-expressing A431 lysate. Like the early endosome-residing Rab5, Rab31 was found mainly in the lighter fractions. However, in the presence of GTPγS and after a pulse-chase with EGF, Rab31 was seen in higher molecular weight fractions, including those where thyroglobulin (a 660kDa dimeric protein) was co-sedimented (Fig. 4.14A). There was, however, no discernible extension of Rab5 to the high molecular weight fractions, even when the blot was overexposed, suggesting that the effect of entering higher molecular weight fractions with GTPγS is specific to Rab31. Interestingly, we observed that the spreading of Rab31 to the heavier fractions occurred at later and not earlier time points of a pulse-chase with EGF, i.e. at 30 and 60 min (Fig. 4.14B). Taken together, the results give some indication that after EGF stimulation, Rab31 becomes associated with a high molecular weight complex that likely contains EGFR, during the time when ligand-bound EGFR is likely to be transferred between early and late endosomes.

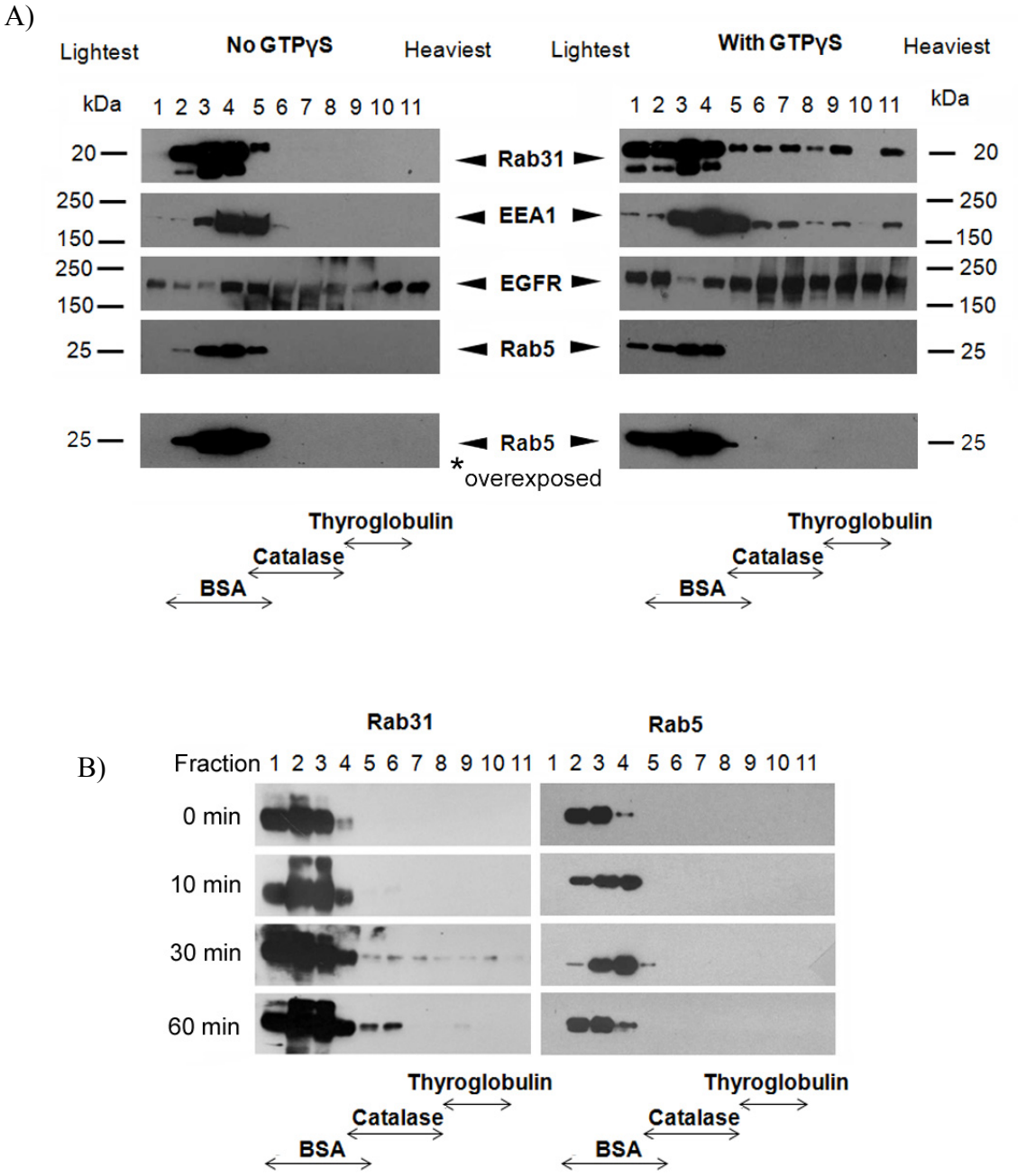


Fig. 4.14. Rab31 associates with a high molecular weight complex
A) 2 mg of A431 Rab31 OE lysates with and without GTPγS loading was resolved by glycerol gradient sedimentation. Fractions were collected, TCA-precipitated and analysed by Western blot for Rab31, EEA1, EGFR, and Rab5. The membrane blot for Rab5 was also overexposed.
B) Lower panel: A431 Rab31 OE cells were pulsed with 0.5 µg/mL EGF and harvested at various time points. 2 mg of lysates were separated by glycerol gradient sedimentation.
The position in the gradient that contains the molecular size markers bovine serum albumin (BSA) (67 kDa), catalase (240 kDa) and thyroglobulin (660 kDa) are indicated.

Rab31-mediated EGFR trafficking occurs downstream of Rab5

Rab5 has been suggested to play a role in both the early and later stages of EGFR trafficking, including internalisation and entry into the early endosome, as well as the transition between the early to late endosome (Barbieri et al., 2000; Dinneen and Ceresa, 2004), with Rab5a being the main isoform involved (Chen et al., 2009). Our results thus far are consistent with Rab31 playing a role in the later stages of EGFR trafficking, probably during transit into late endosomes, by associating with a high molecular weight complex that that likely contains EGFR. We thus sought to determine if there was any interplay between Rab5 and Rab31 in EGFR endocytic transport. We transfected cells with mCherry-Rab5a or Rab31 and quantified the percentage of EGF-FITC puncta positive for Rab5 and Rab31 at various time points post-pulse. We found that Rab5 remains associated with EGF puncta up to 60 min post-pulse, with a slight decrease between 10 and 30 min (Fig. 4.15A). In contrast, we found that the association between Rab31 and EGF only increased significantly at 30 min. Together, the results suggest that while Rab5 appears to associate with both EGFR-carrying early and late endosomes, and may play a role in both early and later stages of EGFR trafficking, Rab31 is more likely to be involved only at the early to late endosome transit stage.

The loss of Rab31 caused a more significant inhibition of colocalisation between ligand-bound EGFR and CD63 than a loss of Rab5, suggesting that Rab5 only plays a partial role in this trafficking step (Fig. 4.15B). A double Rab5a and Rab31 depletion further reduced the percentage of colocalisation. This suggests that while Rab5 may play a role in both the early and later stages of EGFR trafficking, Rab31 is more essential in the later trafficking of ligand-bound EGFR between early and late endosome. This is despite the fairly low percentage of EGF puncta that are

positive for Rab31, which may simply reflect the transient nature of Rab31's association with EGFR-containing membrane structures.

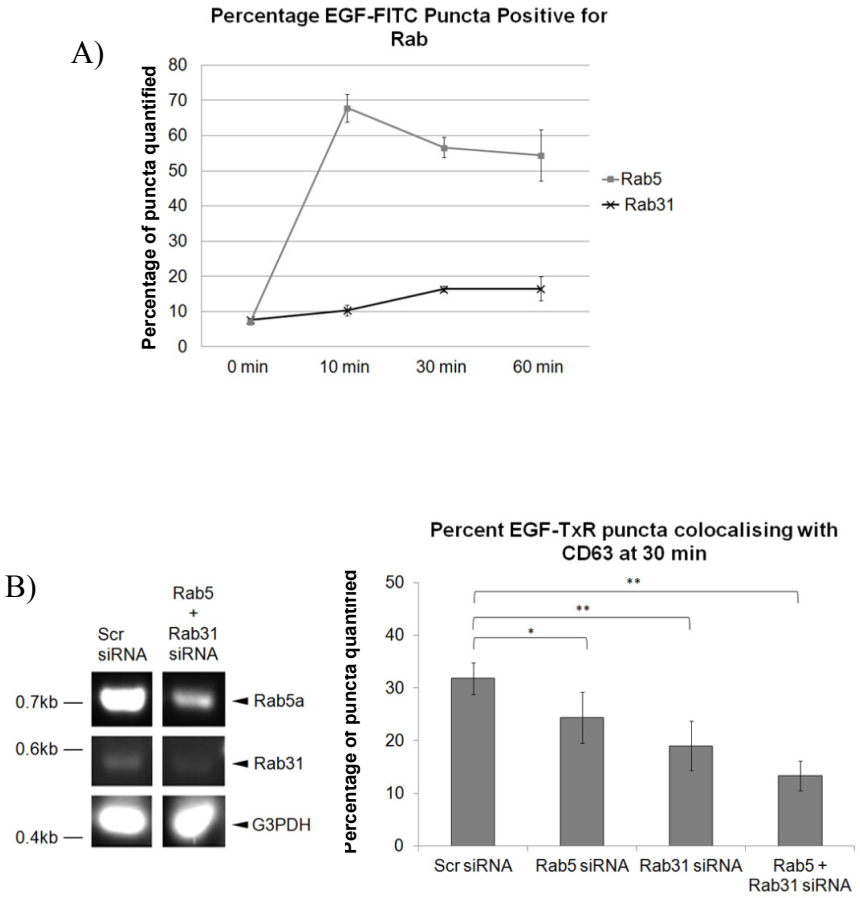


Fig. 4.15. Rab31 appears to act downstream of Rab5 in EGFR trafficking
A) A431 cells were transfected with either mCherry-Rab5a or mCherry-Rab31. Cells were pulsed with 0.5 µg/mL EGF-FITC and fixed at the various time points indicated for immunofluorescence analysis. Percentage of EGF-FITC positive puncta that are positive for either Rab5 or Rab31 was quantified from cells fixed after 0, 10, 30 and 60 min chase, and presented graphically as a percentage of total EGF-FITC puncta counted. 29 cells in 3 independent experiments were analysed and data is shown as mean ± SEM.
B) A431 cells were transfected with scrambled (Scr), Rab5a or Rab31 siRNA as indicated and subsequent analyses were performed after 48 h. *Left panel:* The extent of knockdown was assessed by RT-PCR. Rab5a was depleted by 70% and Rab31 by 75%. *Right panel*: Cells were pulsed with 0.5 µg/mL EGF-TxR and fixed at 30 min. Cells were immunostained for CD63 and the percentages of EGF-TxR puncta that were also positive for CD63 were quantified and presented graphically as a percentage of total EGF-TxR puncta counted. 27 cells in 3 independent experiments were analysed and data is shown as mean ± SEM.*P<0.05 and **P<0.01 by Student's t-test.

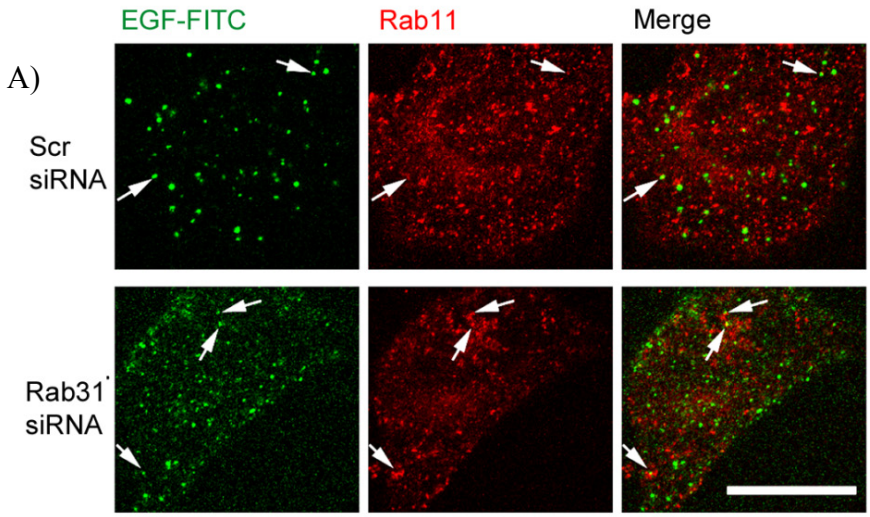
4.3 Results: Rab31 in recycling of EGFR

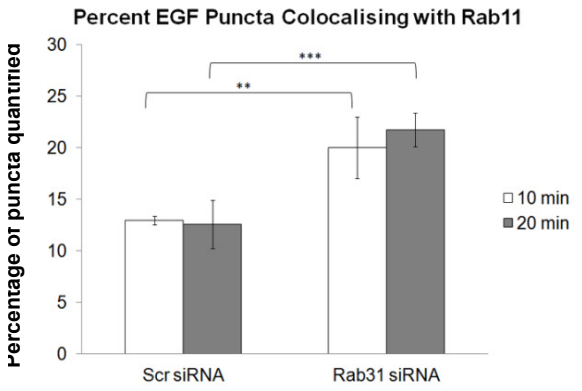
Role of Rab31 in EGFR trafficking is focussed on the degradative, not recycling, pathway

Up to this point, we have focussed on the role of Rab31 in the degradative trafficking pathway of ligand-bound EGFR. Thus far, our data suggests that Rab31 is involved in a later step in EGFR trafficking, after Rab5, and involves the movement of ligand-bound EGFR from early to late endosomes. Notably, though, upon stimulation by EGF, there is also a fraction of the ligand-bound EGFR that is recycled back to the cell surface (Masui et al., 1993; Tong et al., 2013). We investigated how Rab31 might play a role in this process, or if its effect is limited specifically to the degradative pathway.

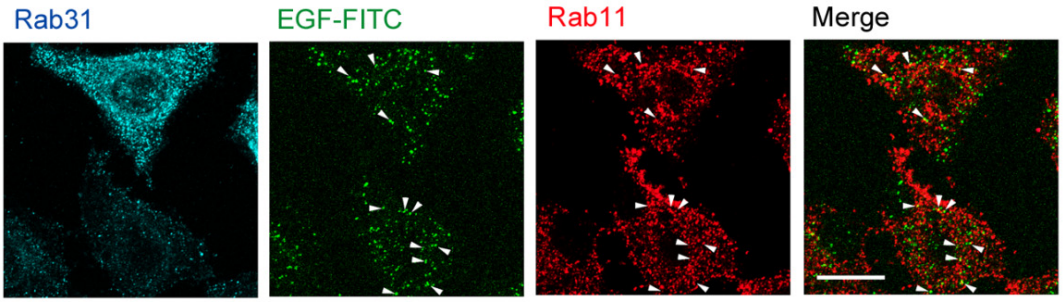
After a pulse with EGF-FITC, we determined the rate of recycling of EGFR in cells as reflected by the percentage of EGF/EGFR puncta colocalising with Rab11, a marker for recycling endosomes. As Rab11 was present at higher levels in HeLa cells compared to A431 (hardly detectable by immunofluorescence), we used HeLa cells for this set of experiments. Depletion of Rab31 significantly increased the percentage of EGF-FITC puncta colocalising with Rab11 at 10 and 20 min, compared to controls (Fig. 4.16A), while overexpression of Rab31 decreased the percentage (Fig. 4.16B). Our results suggest that by enhancing the movement of ligand-bound EGFR from early to late endosomes, Rab31 may channel more ligand-bound EGFR to the degradative pathway, thus indirectly reducing the percentage of EGFR recycled to the cell surface.

A)


EGF-FITC
Rab11
Merge
Scr siRNA
Rab31 siRNA


Percent EGF Puncta Colocalising with Rab11
Percentage of puncta quantified
30
25
20
15
10
5
0
***
**
10 min
20 min
Scr siRNA
Rab31 siRNA

B)


Rab31
EGF-FITC
Rab11
Merge

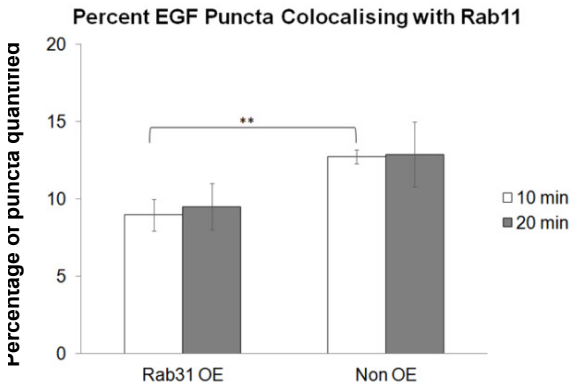

Percent EGF Puncta Colocalising with Rab11
Percentage of puncta quantified
20
15
10
5
0
**
10 min
20 min
Rab31 OE
Non OE

Fig. 4.16. Rab31 indirectly impacts the recycling itinerary of ligand-bound EGFR
A) HeLa cells were transfected with either Scrambled (Scr) or Rab31 siRNA and subsequent assays were performed after 48 h. Cells were pulsed with 0.25 μg/mL EGF-FITC (green), fixed at 20 min, and analysed by colabelling for Rab11 (red). *Upper panel:* Arrows indicate some structures positive for both EGF-FITC and Rab11. Scale bar = 20 μm. *Lower panel:* Percentage of EGF-FITC puncta that are also positive for Rab11 was quantified from cells fixed after 10 and 20 min chase and presented graphically as a percentage of total EGF-FITC puncta counted. 27 cells in 3 independent experiments were analysed and data is shown as mean ± SEM. **P<0.01 and ***P<0.001 by Student's t-test.
B) HeLa cells were transfected with Rab31 (blue), pulsed with 0.25 μg/mL EGF-FITC (green) and fixed at 20 min for analysis by co-immunostaining for Rab11 (red). *Upper panel:* Arrows indicate some structures positive for both EGF-FITC and Rab11. Scale bar = 20 μm. *Lower panel:* Percentage of EGF-FITC puncta that are positive for Rab11 was quantified from cells fixed after 10 and 20 min chase and presented graphically as a percentage of total EGF-FITC puncta counted. 27 cells in 3 independent experiments were analysed and data is shown as mean ± SEM. **P<0.01 by Student's t-test.

4.4 Chapter Discussion: Rab31 plays a role in early-to-late endosome trafficking of ligand-bound EGFR through a trafficking complex

We have expanded our previous understanding of the role of Rab31 in EGFR trafficking by showing that Rab31 is likely to participate at the stage when ligand-bound EGFR traffics from early to late endosome, by forming a trafficking complex with EGFR. Currently, how Rab31 factors in the EGFR trafficking process in comparison to other Rabs involved has not yet been fully defined. Our results point to the possibility that Rab31 acts downstream of the initial internalisation of EGFR that is coordinated by Rab5. It is pertinent to note here that Rab5, in some studies, has also been shown to be involved in the trafficking step between early and late endosome. It is not entirely clear to what extent Rab5 is essential for this step, but evidence suggests that loss of Rab5 does not completely block the trafficking (Chen et al., 2009; Dinneen and Ceresa, 2004). Moreover, here we have shown that Rab31 depletion causes a more severe disruption in the trafficking to late endosomes

compared to Rab5 depletion. At the very least, Rab31 can be said to function alongside Rab5 in the trafficking of ligand-bound EGFR.

There are many instances in literature of the interplay between various Rabs in the ordering of transport steps. For example, Rab5 and Rab7 have been shown to coordinate the maturation of endosomes via a process termed Rab conversion, when the preceding Rab engages various proteins that recruit the subsequent Rab (Rink et al., 2005). How and when the various Rabs coordinate EGFR-related traffic remains to be fully elucidated. Renzis et al. described the divalent effector Rabenosyn5, which binds both Rab5 and Rab4 simultaneously, enabling Rab5 and Rab4 membranes to interact, thus allowing the transition between early endosomes and fast recycling endosomes (De Renzis et al., 2002). It is thus likely that other interacting proteins facilitate the Rab31-mediated transition of ligand-bound EGFR between the early and late endosome. Having seen that Rab31 is part of a trafficking complex that is likely to include EGFR, we thus sought to determine which other interacting partners of Rab31 might participate in its role in EGFR trafficking. This is explored further in the following chapter.

5. The role of Rab31-interacting proteins in Rab31-mediated EGFR trafficking

5.1 Chapter Introduction: Potential Rab31-interacting proteins in an EGFR-trafficking complex

As discussed in Section 4.4, Rab31 is part of a trafficking complex that is likely to include EGFR, and plays a role in the early-to-late endosome transition. At the same time, other interacting proteins of Rab31 are likely to be part of this trafficking complex, and may mediate the transition between early and late endosome. EEA1, as a common effector for both Rab5 and Rab31, might also fulfil this role, as it has two Rab binding domains; Rab5 has greater affinity for the N-terminal domain, whereas Rab31 has equal affinity for both (Lodhi et al., 2007). Our glycerol gradient sedimentation results suggest that the trafficking complex is of high molecular weight and likely includes more than just EGFR. In the glycerol gradient sedimentation of the A431 lysate, we observed that along with Rab31, EEA1's presence also appeared to be extended to the higher molecular weight fractions upon EGF pulse (Fig. 4.13). We therefore sought to take a closer look at the role of EEA1 in Rab31-mediated EGFR trafficking.

We also chose to explore the role of GAPex5 in Rab31-mediated EGFR trafficking. GAPex5 contains a Ras-GAP domain and also a VPS9 GEF domain, and has been identified to bind to EGFR through Cbl, an E3 ubiquitin ligase, upon receptor dimerisation (Su et al., 2007). We have shown in Section 3.3 that depletion of GAPex5 impacts the subcellular localisation of Rab31. We therefore sought to investigate if this also had subsequent effects on the trafficking of ligand-bound EGFR.

5.2 Results: Role of EEA1 in Rab31-mediated EGFR trafficking

EEA1 is part of the Rab31-EGFR trafficking complex and plays a role in Rab31-EGFR association

Using immunofluorescence analysis of A431 cells stably expressing EGFP-Rab31, we observed that after a pulse-chase with EGF-TxR, a portion of the EEA1 puncta colocalised with Rab31, together with EGF-TxR (Fig. 5.1A). This percentage increased gradually and was significant after a 30 min chase with EGF (Fig. 5.1B). This mirrors the colocalisation data between Rab31 and EGFR, and suggests that EEA1 may be associated together with Rab31 and EGFR, likely in high molecular weight trafficking complexes.

We further probed the role of EEA1 in the Rab31-EGFR association. With a loss of EEA1, GST-Rab31 was unable to pulldown EGFR, even with the presence of GTPγS (Fig. 5.2A). This suggests that EEA1 must be present for the interaction between Rab31 and EGFR to occur. (We should note that while EEA1 interaction itself was not detected here, we were able to detect GST-Rab31 pulldown of EEA1 if cells were first pulsed with EGF before harvesting of cell lysate for the assay (Fig. 5.2B)). Furthermore, after a 30 min pulse-chase with EGF-TxR, there was a delocalisation between EGFP-Rab31 and EGF-TxR when EEA1 was silenced, compared to cells in the control population (Fig. 5.3). This supports our postulation that EEA1 is important for the interaction between Rab31 and EGFR in the trafficking complexes.

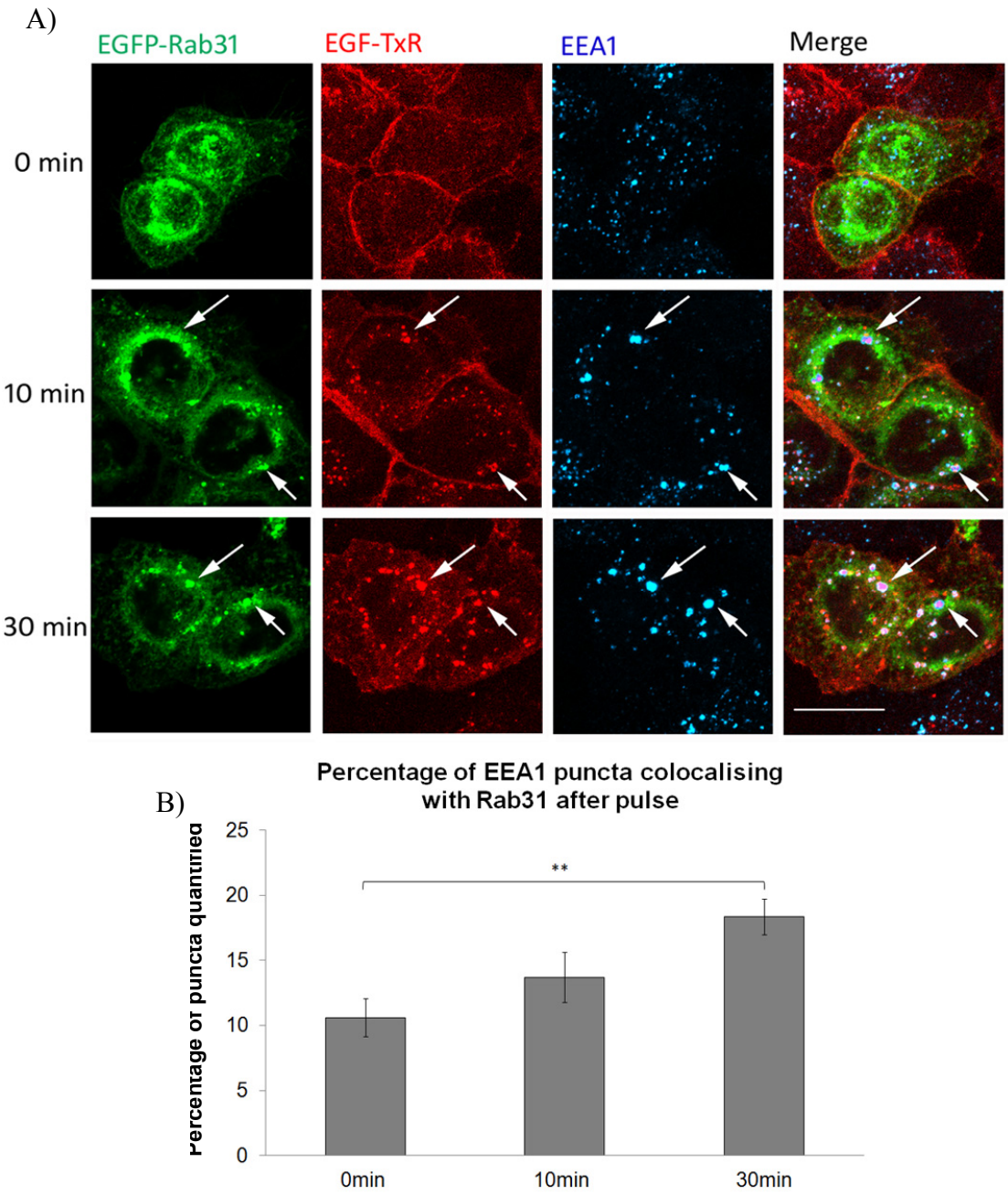


Fig. 5.1. EEA1 colocalises with Rab31 and EGFR
*A)*A431 cells stably expressing EGFP-Rab31 (green) were pulsed with 0.5 µg/mL EGF-TxR (red), fixed at various time points, and co-labelled using EEA1 antibodies (pseudo-coloured blue). Arrows indicate some structures positive for EGFP-Rab31, EGF-TxR and EEA1. Scale bar = 20 µm.
B) Percentage of EEA1 puncta that are positive for Rab31 was quantified from cells fixed after 0, 10 and 30 min chase and presented graphically as a percentage of total EGF-TxR puncta counted. 33 cells in 3 independent experiments were analysed and data is shown as mean ± SEM. **P<0.01 by Student's t-test.

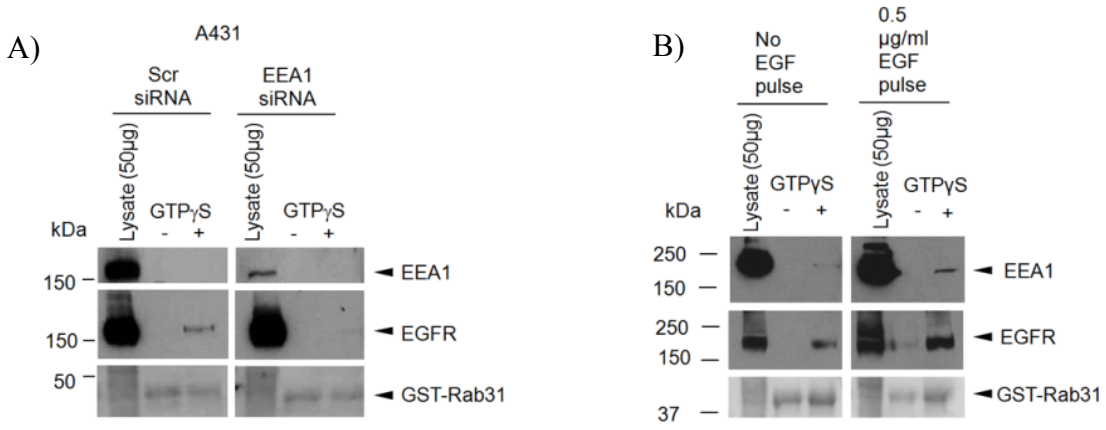


Fig. 5.2. EEA1 associates with Rab31 and EGFR
A) A431 cells were transfected with Scrambled (Scr) or EEA1 siRNA. 1 mg lysates with and without GTPγS were incubated with 20 µg GST-Rab31 and glutathione beads. The ability of GST-Rab31 to affinity pulldown EGFR was analysed by Western blot. Ponceau S staining of the GST proteins used is shown.
B) Cells were pulsed with 0.5 µg/mL EGF before harvesting after a 30 min chase. 1 mg lysates with and without GTPγS were incubated with 20 µg GST-Rab31 and glutathione beads. Ability of GST-Rab31 to affinity pulldown EGFR and EEA1 was analysed by Western blot. Ponceau S staining of the GST proteins used is shown.

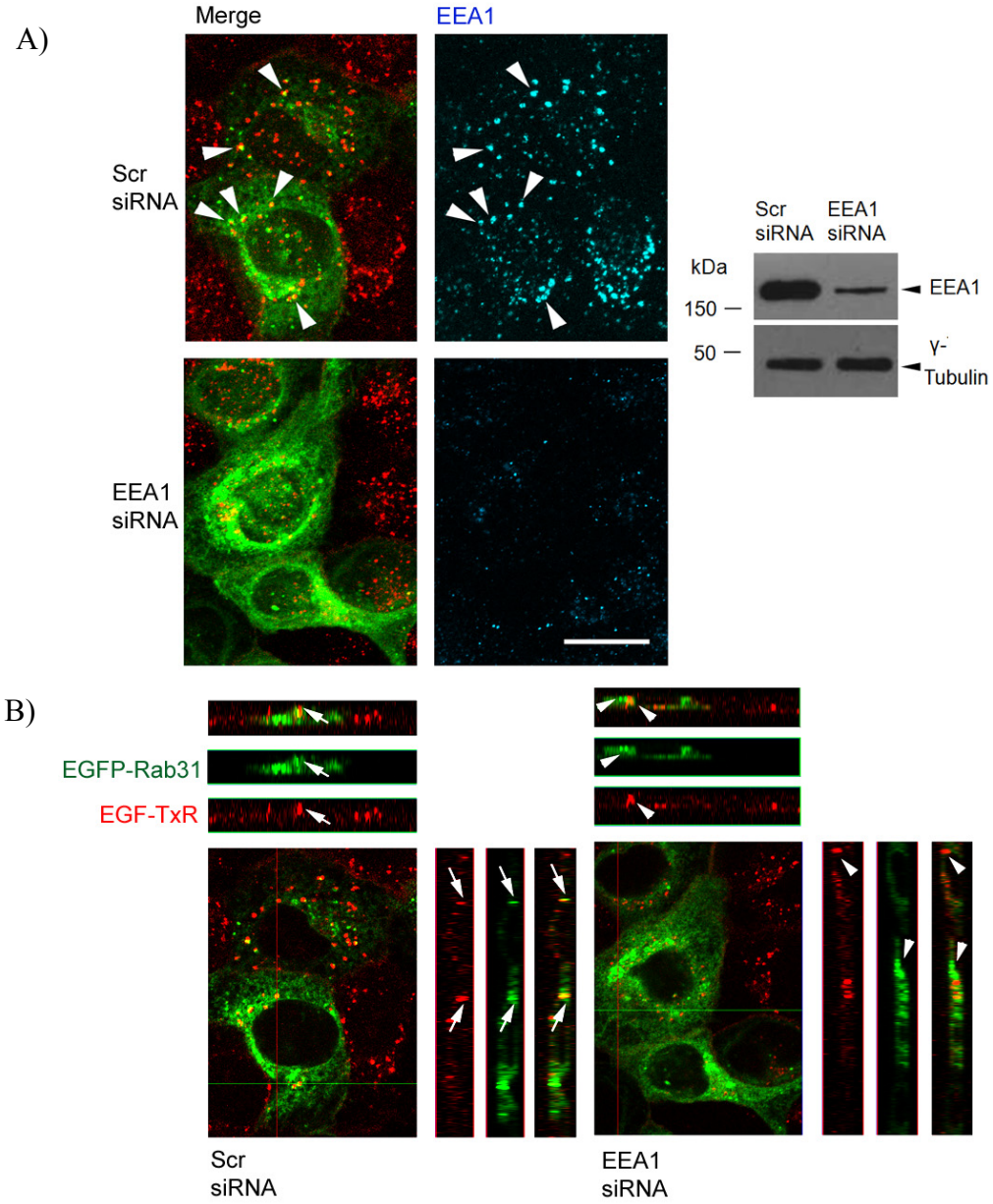

A)
Merge
EEA1
Scr siRNA
EEA1 siRNA
Scr siRNA
EEA1 siRNA
kDa
150
50
EEA1
γ-Tubulin
B)
EGFP-Rab31
EGF-TxR
Scr siRNA
EEA1 siRNA
C)
Percentage Colocalisation of EGF puncta with Rab31 after EGF Pulse
Percentage of puncta quantified
20
15
10
5
0
**
0min
10min
30min
Scr
EEA1 siRNA

Fig. 5.3. Depletion of EEA1 results in delocalisation between Rab31 and EGFR
A431 cells stably expressing EGFP-Rab31 (green) were transfected with Scr or EEA1 siRNA. Cells were pulsed 48 h later with 0.5 μg/mL EGF-TxR (red), fixed after 30 min, and co-labelled for EEA1 (pseudo-coloured blue).
A) Knockdown of EEA1 was assessed by immunofluorescence (left) and Western blot (right). EEA1 was depleted by 80%. Arrowheads also indicate points of colocalisation between EGFP-Rab31, EGF-TxR and EEA1 puncta. Scale = 20 μm.
B) Orthogonal projection of the 3D stacked confocal images shown in *(A)*. In cells transfected with the scrambled siRNA there is colocalisation between EGFP-Rab31 (green) and EGF-TxR (red) (arrows). In cells with EEA1 knockdown, EGFP-Rab31 and EGF-TxR appeared delocalised (arrowheads).
C) Percentage of EGF-TxR positive puncta that are also positive for EGFP-Rab31 was quantified from Scr and EEA1 siRNA transfected cells fixed after 0, 10 and 30 min chase, and graphically represented as a percentage of total EGF-TxR puncta counted. 29 cells in 3 independent experiments were analysed and data is shown as mean ± SEM. **P<0.01 by Student's t-test.

We next looked at how the loss of EEA1 might affect the Rab31-mediated enhancement of endocytic trafficking of ligand-bound EGFR. When EEA1 was silenced, there appeared to be little difference in the size of EGF puncta between cells overexpressing Rab31 (asterisks) and non-overexpressing cells (Fig. 5.4), unlike cells in the control population. Quantification of this phenomenon showed that there was significantly lower percentage of puncta <0.05 μm in cells overexpressing Rab31 in the Scr siRNA transfected population, whereas this difference was lost in the EEA1-silenced population. We also quantified the percentage of EGF-TxR puncta that were positive for CD63 (Fig. 5.5), and found that in the control population, there was a significantly higher percentage of colocalisation in cells with Rab31 overexpression (Box A), compared to cells without overexpression (Box B). In contrast, in EEA1-silenced cells, there was no significant difference between Rab31-overexpressing and non-overexpressing cells. This suggests that the Rab31-mediated effect on EGFR trafficking to the CD63 late endosome compartment is abrogated with a loss of EEA1. Overall, our results suggest that Rab31 associates with ligand-bound EGFR and enhances its trafficking, and that this association requires the presence of EEA1.

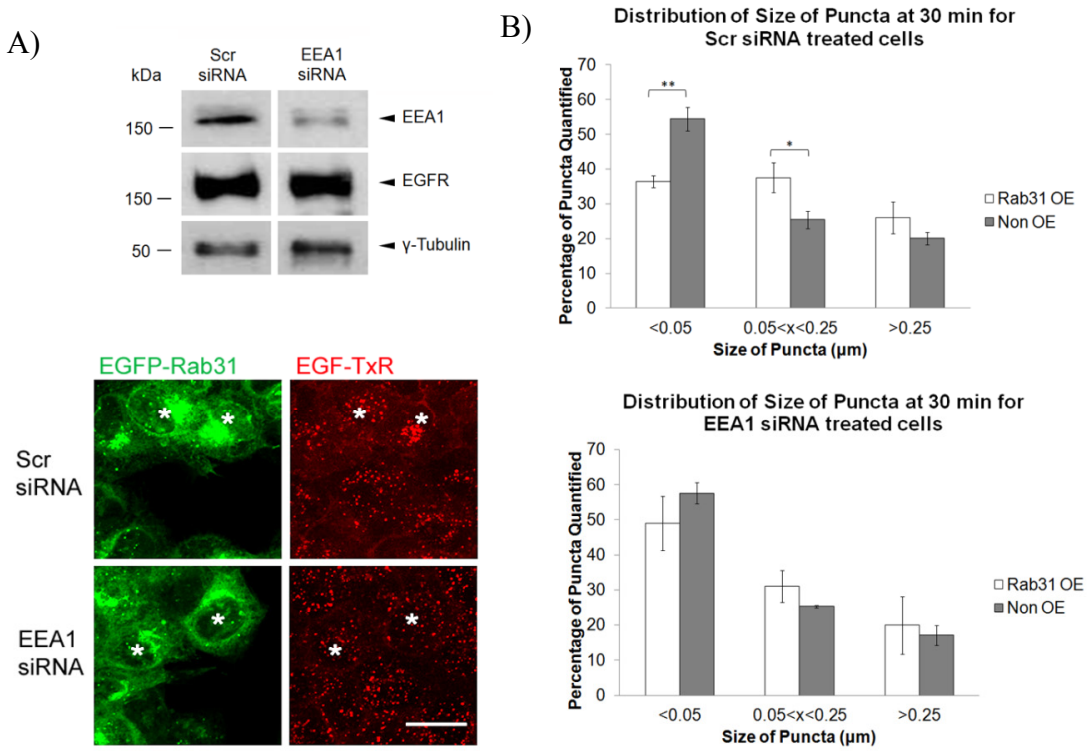


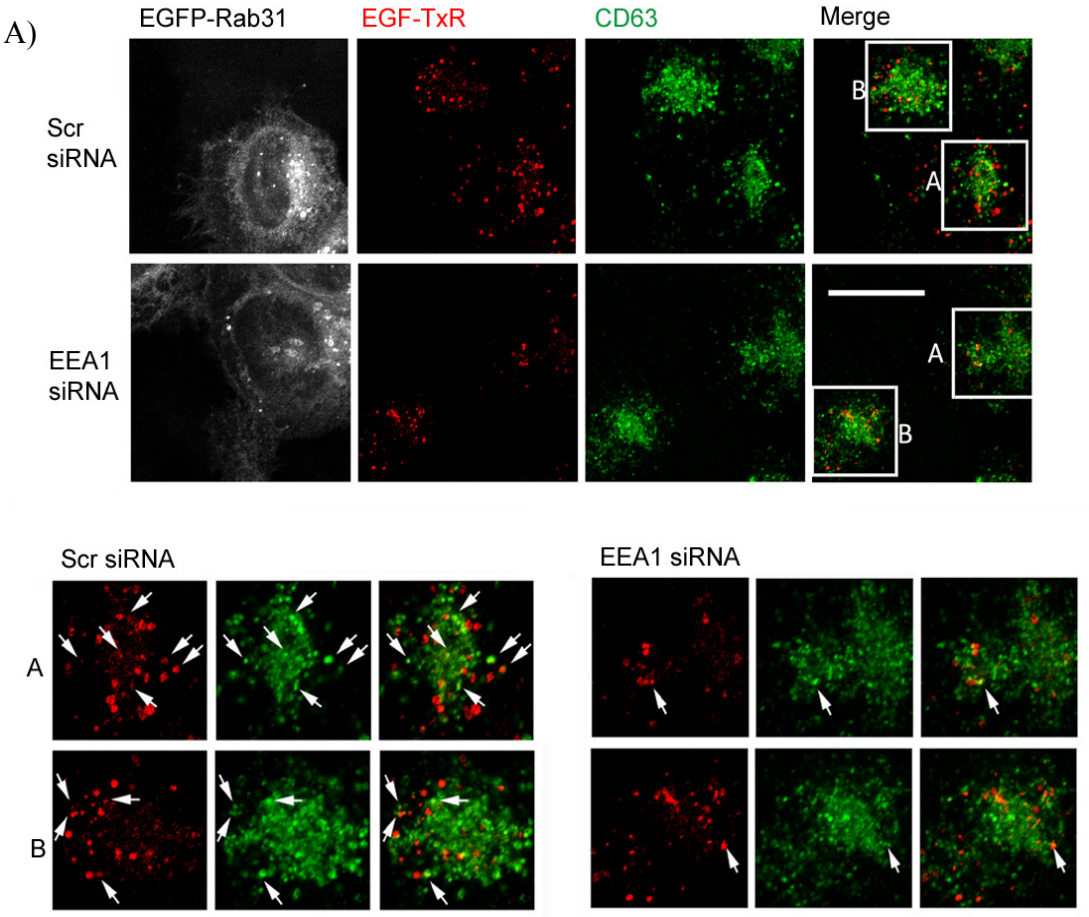
Fig. 5.4. EEA1 is important for the Rab31-mediated enhancement of ligand-bound EGFR endocytic trafficking

A431 cells stably expressing EGFP-Rab31 were transfected with Scr or EEA1 siRNA and analysed after 48 h.

A) The levels of EEA1 and EGFR were analysed by Western blot. EEA1 was depleted by 80%. Cells were pulsed with 0.5 μg/mL EGF-TxR (red), and fixed at 30 min for comparison of the puncta sizes between cells overexpressing EGFP-Rab31 (green, asterisks) and non-overexpressing cells in the same populations. Scale bar = 20 μm.

B) Sizes of EGF-TxR puncta in Rab31 overexpressing (OE) and non-overexpressing cells from the Scrambled (upper graph) or EEA1 siRNA-treated populations (lower graph) were quantified using ImageJ, and the size distribution is represented graphically as a bar chart. 19 cells in 3 independent experiments were analysed and data is shown as mean ± SEM. *P<0.05 and **P<0.01 by Student's t-test.

A)


EGFP-Rab31
EGF-TxR
CD63
Merge
Scr siRNA
EEA1 siRNA
B
A
A
B
Scr siRNA
EEA1 siRNA
A
B

B)

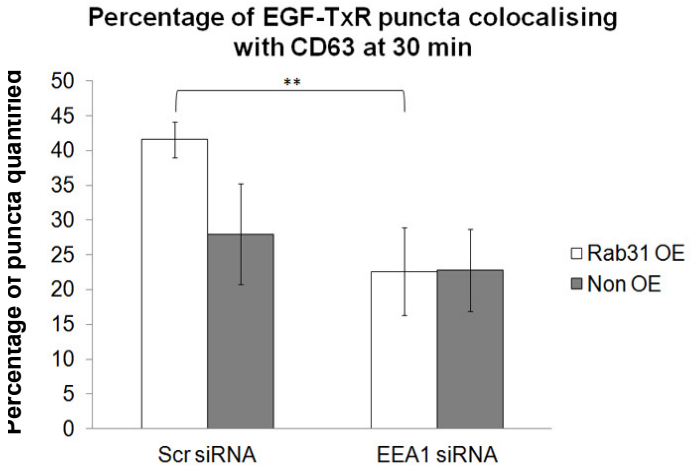

Percentage of EGF-TxR puncta colocalising with CD63 at 30 min
Percentage of puncta quantified
50
45
40
35
30
25
20
15
10
5
0
**
Scr siRNA
EEA1 siRNA
Rab31 OE
Non OE

Fig. 5.5. Depletion of EEA1 reduces entry of ligand-bound EGFR into late endosome
A) Scr and EEA1 siRNA treated cells were immunostained for CD63 (pseudo-coloured green), along with EGF-TxR (red) and EGFP-Rab31 (pseudo-coloured white). The lower panel shows individual and merged fluorescence signals of the boxed areas, magnified 2x. Box A represents cell with Rab31 overexpression while Box B represents cells with no overexpression. Arrows indicate some structures positive for both EGF-TxR and CD63. Scale bar = 20 μm.
B) The percentage of EGF-TxR puncta that are positive for CD63 were quantified and graphically represented as a percentage of total EGF-TxR puncta counted. 36 cells in 3 independent experiments were analysed and data is shown as mean ± SEM. **P<0.01 by Student's t-test.

5.3 Results: Role of GAPex5 in Rab31-mediated EGFR trafficking

The Rab31 GEF GAPex5, but not RIN3, plays a role in the Rab31-EGFR trafficking complex

GAPex5 is a guanine nucleotide exchange factor (GEF) for Rab31, and thus acts to activate Rab31 by enhancing the exchange of GDP for GTP. It has also been shown to be involved in the early steps of ligand-bound EGFR internalisation by interacting with Cbl, which itself binds to EGFR upon stimulation with the ligand (Su et al., 2007). As such, GAPex5 stands as a good candidate for a possible mediator of the formation of the Rab31-EGFR trafficking complex.

In our initial studies with depletion of GAPex5 in Rab31-overexpressing A431 cells, we observed that loss of GAPex5 resulted in a distinct dispersal of Rab31 from the TGN, as seen by a delocalisation with the trans-Golgi marker TGN46 (Fig. 3.4). This effect was specific to GAPex5, as depletion of another GEF of Rab31, RIN3 (Kajiho et al., 2011), did not bring about a similar effect in A431 cells. The dispersal of Rab31 from the TGN did not result in its corresponding accumulation in other vesicular compartments, which indicated that the membrane association of Rab31 was lost.

We thus looked at how GAPex5 depletion would affect the Rab31-EGFR interaction. Loss of GAPex5 resulted in a reduced pulldown of EEA1 and EGFR by GST-Rab31, even in the presence of GTPγS (Fig. 5.6A, B). Interestingly, as with the dispersal of Rab31 from the TGN, this effect was specific to GAPex5, as RIN3 depletion did not produce the same result (Fig. 5.6C). This suggests that reduced Rab31-EGFR interaction seen with a GAPex5 knockdown was not due to reduction in Rab31 activation resulting from a general loss of GEF activity, but was rather a GAPex5 specific effect.

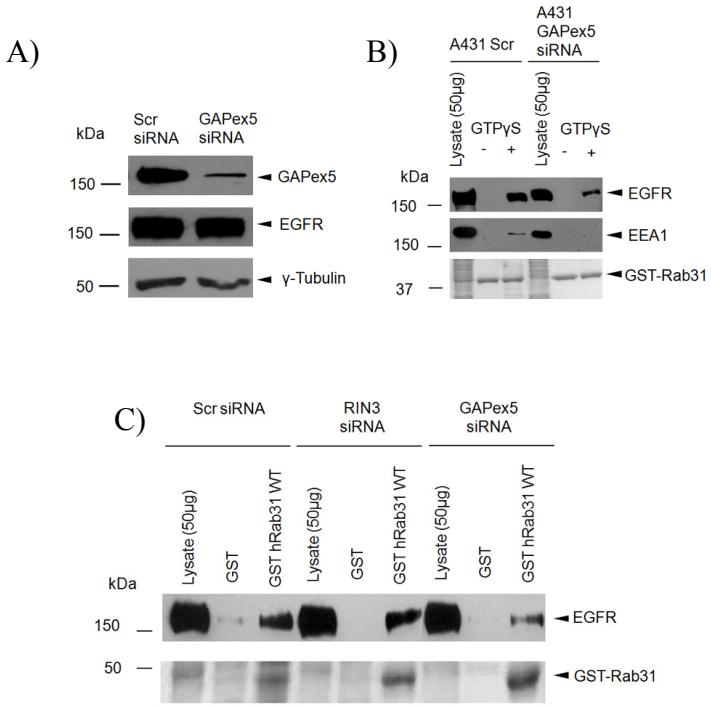


Fig. 5.6. Depletion of GAPex5 abrogates Rab31-EGFR association
A) A431 cells were transfected with GAPex5 siRNA and harvested 48 h later. The extent of GAPex5 knockdown and the levels of EGFR were assessed by Western blot. GAPex5 was depleted by 80%.
B) GST-Rab31 affinity pulldown assay was performed with the harvested lysates. Cells were pulsed with 0.5 µg/mL EGF before harvesting after a 30 min chase. 1 mg lysates with and without GTPγS were incubated with 20 µg GST-Rab31 and glutathione beads. Ability of GST-Rab31 to affinity pulldown EGFR and EEA1 was analysed by Western blot. Ponceau S staining of the GST proteins used is shown.
C) 1 mg of A431 cell lysate harvested 48 h after transfection with relevant siRNA were incubated with 20 µg GST or GST-Rab31 and glutathione beads, in the presence of GTPγS, and the ability of the fusion proteins to pulldown EGFR was analysed by Western blot. GST proteins were visualised with Ponceau S stain.

As with EEA1 depletion, the loss of GAPex5 abrogated Rab31-mediated enhancement of ligand-bound EGFR trafficking. In the population of control cells, those overexpressing EGFP-Rab31 (asterisks) had larger EGF-TxR puncta 30 min post-pulse, compared to those without overexpression. This difference was not observed in the population of GAPex5-silenced cells (Fig. 5.7). Quantification of this phenomenon showed that there was significantly higher percentage of puncta >0.25 μm in cells overexpressing Rab31 in the Scr siRNA transfected population, whereas this difference was lost in cells in the GAPex5-silenced population. Also, in the control population, there was a significantly higher percentage of colocalisation between EGF-TxR and CD63 in cells with Rab31 overexpression (Box A), compared to cells without overexpression (Box B) (Fig. 5.8). In contrast, in the GAPex5-silenced population, there was no significant difference between Rab31-overexpressing and non-overexpressing cells. Together, the results suggest that the Rab31 GEF GAPex5, but not RIN3, may have a specific role in Rab31-EGFR trafficking, in bringing together the interaction between Rab31, EGFR and EEA1 in the trafficking complex.

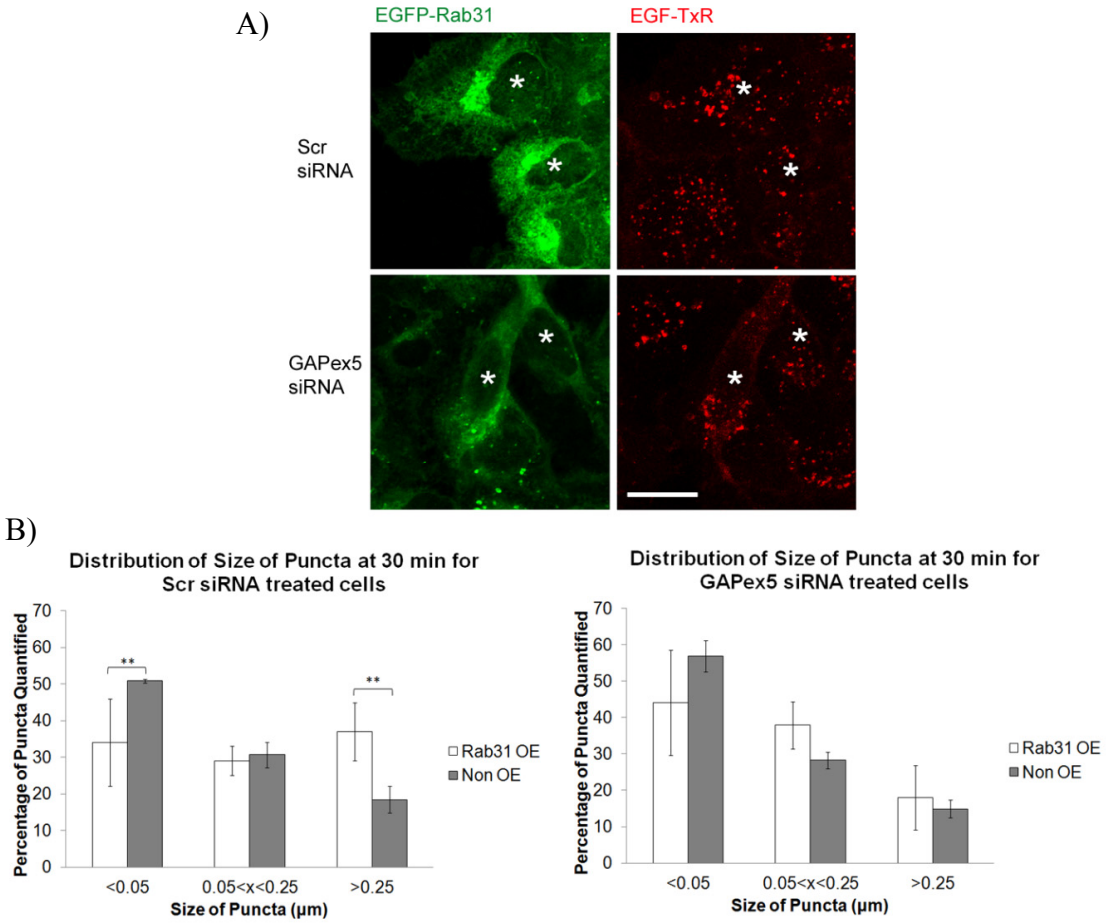
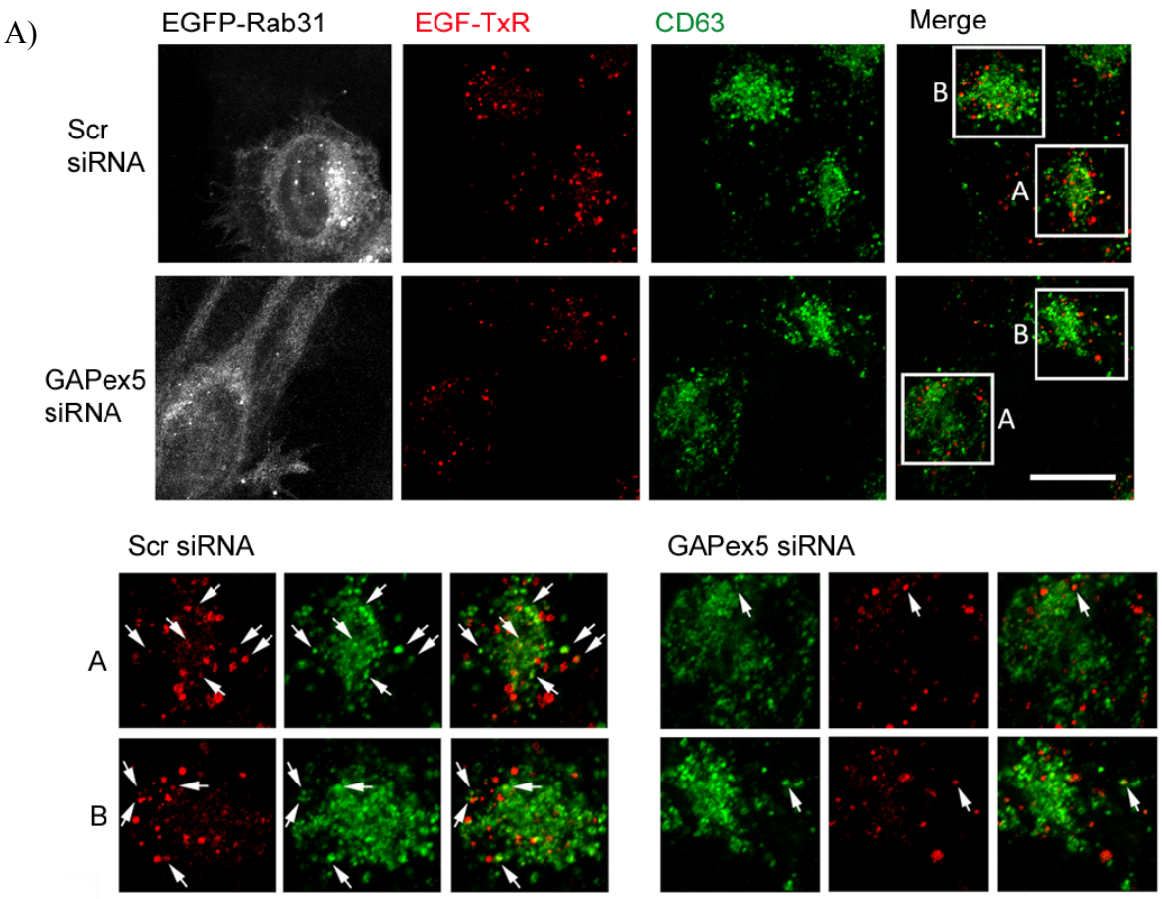


Fig. 5.7. GAPex5 is important for the Rab31-mediated enhancement of ligand-bound EGFR endocytic trafficking

A) A431 cells stably expressing EGFP-Rab31 (green) were transfected with Scrambled (Scr) or GAPex5 siRNA. Cells were pulsed with 0.5 µg/mL EGF-TxR and fixed at 30 min for analysis of the size of EGF-TxR (red) puncta in cells that do (asterisks) or do not overexpress EGFP-Rab31. Scale bar = 20 µm.

B) Sizes of EGF-TxR puncta in Rab31 overexpressing (OE) and non-overexpressing cells from the Scr (left graph) or GAPEx5 siRNA-treated population (right graph) were quantified using ImageJ, and the size distribution is represented graphically as a bar chart. 21 cells in 3 independent experiments were analysed and data is shown as mean ± SEM. **P<0.01 by Student's t-test.


A)
EGFP-Rab31
EGF-TxR
CD63
Merge
Scr siRNA
GAPex5 siRNA
B
A
Scr siRNA
GAPex5 siRNA
A
B

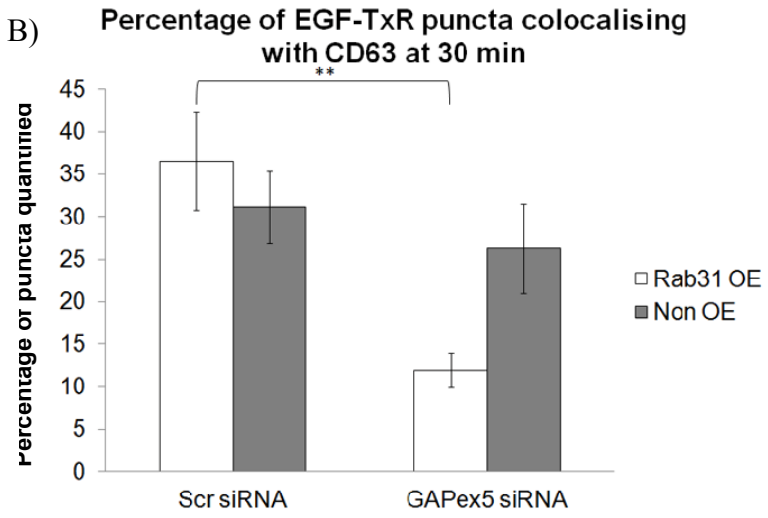

B)
Percentage of EGF-TxR puncta colocalising with CD63 at 30 min
**
Percentage of puncta quantified
45
40
35
30
25
20
15
10
5
0
Scr siRNA
GAPex5 siRNA
Rab31 OE
Non OE

Fig. 5.8. Depletion of GAPex5 hinders entry of ligand-bound EGFR into late endosome
A) Scr and GAPex5 siRNA treated cells were immunostained for CD63 (pseudo-coloured green), along with EGF-TxR (red) and EGFP-Rab31 (pseudo-coloured white). The lower panel shows individual and merged fluorescence signals of the boxed areas, magnified 2x. Box A encloses the central area of cells with Rab31 overexpression while Box B encloses central areas of cells with no Rab31 overexpression. Arrows indicate some structures positive for both EGF-TxR and CD63. Scale bar = 20 μm.
B) The percentage of EGF-TxR puncta that are also positive for CD63 were quantified and are graphically presented as a percentage of total EGF-TxR puncta counted. 41 cells in 3 independent experiments were analysed and data is shown as mean ± SEM. **P<0.01 by Student's t-test.

5.4 Results: RIN3 mediates a separate trafficking role of Rab31

In our investigation into the effect of depletion of RIN3, we observed that there was no significant effect on trafficking of EGFR. However, we observed a separate phenomenon affecting the localisation of M6PR.

The cation dependent and cation independent mannose 6-phosphate receptors (M6PR) are transmembrane receptors which recognise the mannose 6-phosphate post-translational modification on newly synthesised proteins such as acid hydrolases at the TGN. These serve to transport the acid hydrolases to the endosomal network. In the acidified environment, M6PRs release their cargo and are recycled to the TGN, while the cargo is subsequently trafficked to lysosomes, where they serve their function. M6PR thus cycles between the TGN and the endosomal network (Damen et al., 2006).

The loss of RIN3, in cells overexpressing Rab31, resulted in a dispersed localisation of M6PR. This was not seen in a knockdown of GAPex5 (Fig. 5.9A, B). The overall protein levels of M6PR did not appear severely affected (Fig. 5.9C). We also looked at the effect on another late endosomal marker, CD63, and saw no effect, nor did we observe an effect on the early endosome marker EEA1 (Fig 5.10). This

indicated that the effect was not a general disruption of endocytic trafficking pathways or markers. Also, the effect of RIN3 depletion on M6PR was not merely due to the loss of a functional active Rab31, since the phenomenon was only seen in cells with Rab31 overexpression. Moreover, depletion of Rab31 did not recapitulate the phenomenon that we observed (Fig. 5.11A). Also, the effect was only observed in cells overexpressing Rab31, but not another Rab from the Rab5 subfamily, Rab22, which is implicated in early endosomal trafficking (Fig. 5.11B). Taken together, our results suggest that the loss of RIN3 affects the localisation of M6PR specifically, in cells with high levels of Rab31.

Our results suggest that Rab31 may play a role in multiple trafficking events within the cell. This, in turn, is likely to be determined in part by the localisation and specificity of the GEFs which activate Rab31.

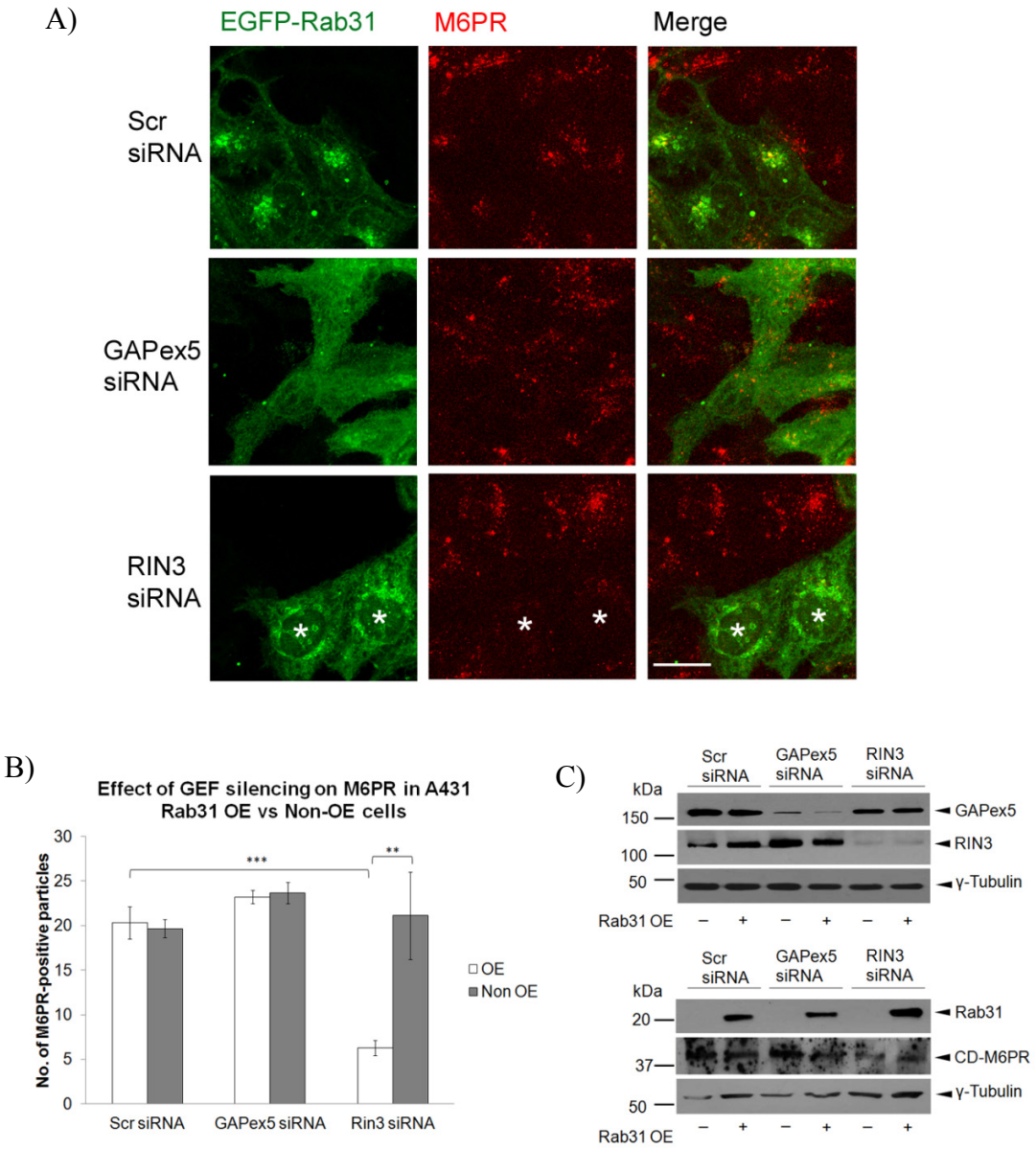


Fig. 5.9. Depletion of RIN3 results in disruption to the localisation of M6PR
A431 cells stably expressing EGFP-Rab31 (green) were transfected with Scr, GAPex5 or RIN3 siRNA and analysed after 48 h.
A) Cells were immunostained for M6PR (red). Asterisks indicate cells with Rab31 overexpression and dispersed M6PR staining. Scale bar = 20 μm.
B) Number of M6PR-labelled particles was quantified by ImageJ. 43 cells in 3 independent experiments were analysed and data is shown as mean ± SEM. **P<0.01 by Student's t-test.
C) Cell lysate was also harvested and analysed by Western blot for the proteins indicated. GAPex5 and RIN3 were depleted by 80%.

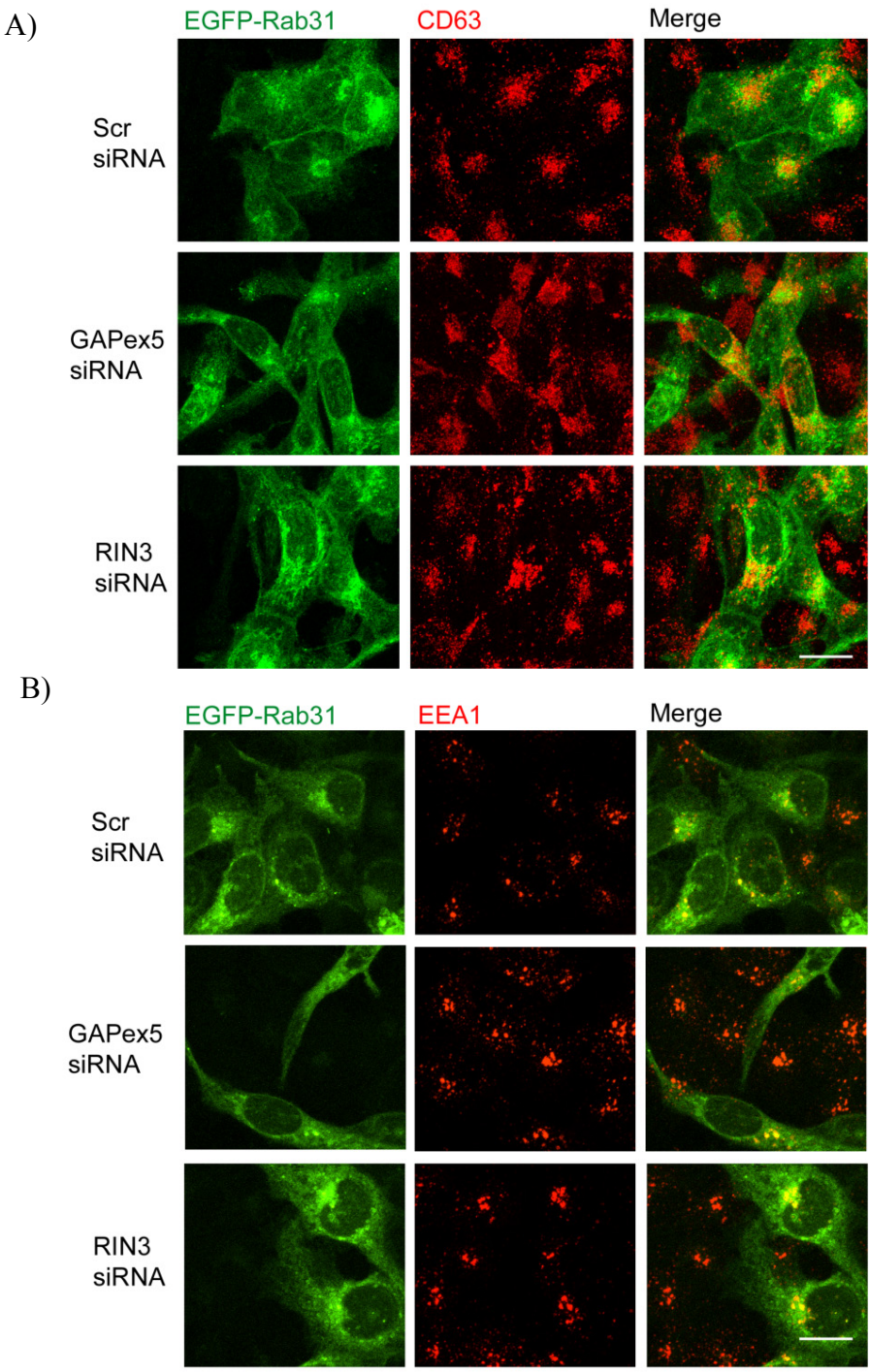


Fig. 5.10. Depletion of RIN3 does not affect CD63 or EEA1 localisation
A431 cells stably expressing EGFP-Rab31 (green) were transfected with Scr, GAPex5 or RIN3 siRNA and analysed after 48 h.
A) Cells were immunostained for CD63 (red).
B) Cells were immunostained for EEA1 (red).
Scale bar = 20 μm.

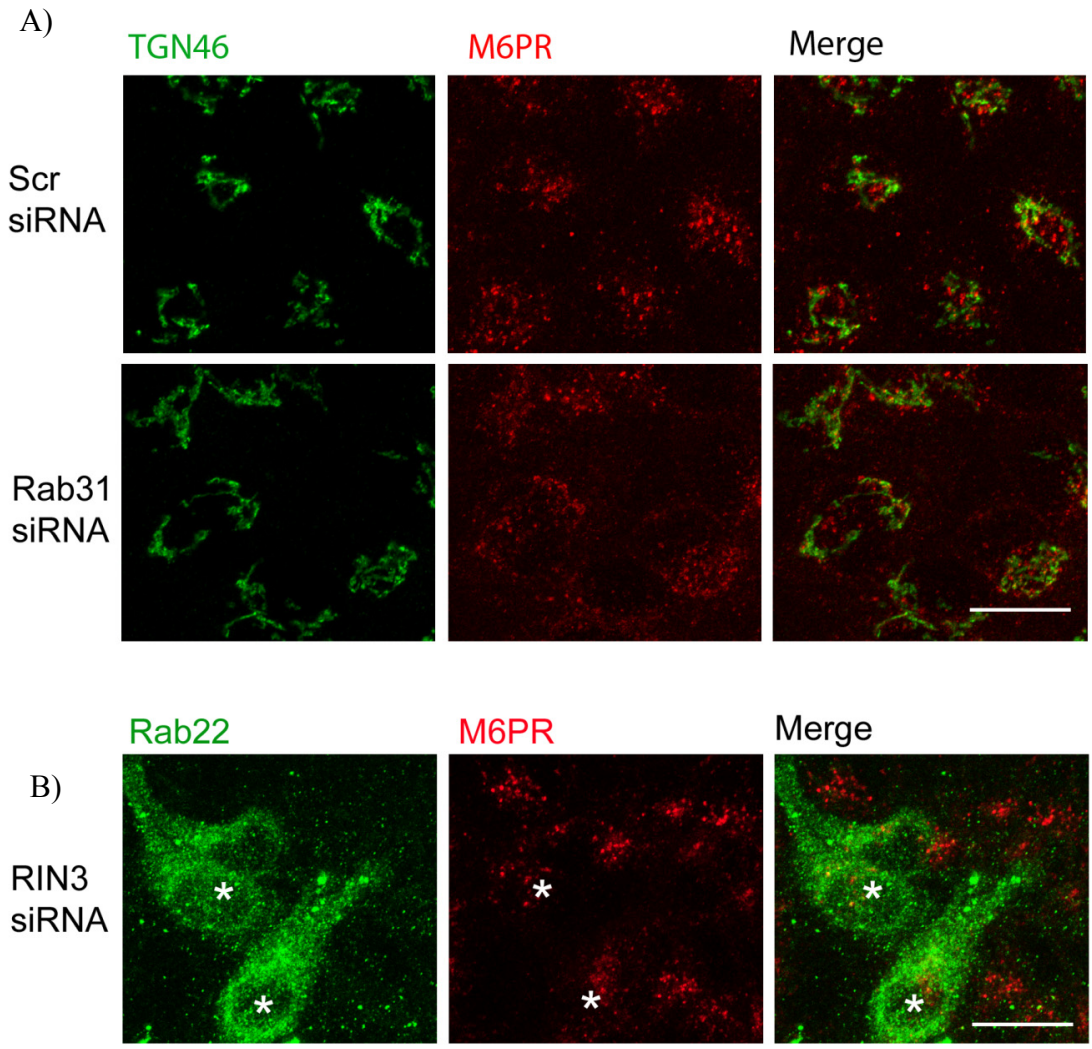


Fig. 5.11. Effect of RIN3 depletion on M6PR is specific to cells with Rab31 overexpression
A) A431 cells were transfected with Scr or Rab31 siRNA and analysed after 48 h. Cells were fixed and immunostained for TGN46 (green) and M6PR (red).
B) A431 cells were transfected with Rab22 expression vector and RIN3 siRNA and analysed after 48 h. Cells were fixed and immunostained for Rab22 (green, asterisks) and M6PR (red).
Scale bar = 20 μm.

5.5 Chapter Discussion: Interacting proteins mediate different roles of Rab31

Role of EEA1 in Rab31-EGFR trafficking complex

We have shown in Section 5.2 that EEA1 colocalised with Rab31 puncta upon EGF stimulation. When EEA1 expression was silenced, the interaction between Rab31 and EGFR was lost, and the effect of Rab31 overexpression on ligand-bound EGFR trafficking was also attenuated. We thus postulate that Rab31 might be mediating its effect on trafficking of EGFR through an EEA1-containing trafficking complex.

To date, evidence for the role of EEA1 in endocytosis of EGFR has been varied, with some evidence pointing towards EEA1 being dispensable, at least in the early step of clathrin-mediated internalisation of EGFR from the cell surface (Chen and Wang, 2001; Huang et al., 2004). Likewise, in our hands, depletion of EEA1 did not abrogate the initial internalisation of EGFR. What our results do suggest that EEA1 is directly involved in the interaction between Rab31 and EGFR, and is important for Rab31-regulated trafficking of EGFR between early and late endosomes. It is tantalizing to speculate that Rab31 might perhaps be recruited to the EGFR trafficking complex subsequent to the involvement of Rab5, which may be responsible for first engaging EEA1 onto EGFR-carrying endosomes.

Role of a GEF in Rab31/EEA1-mediated EGFR trafficking complex

We have previously shown that loss of GAPex5 phenocopied Rab31 depletion (Ng et al., 2009). Here, we have further shown that overexpression of Rab31 is unable to rescue a loss of GAPex5. One reason for this is likely to be the reduced activation of Rab31 due to a lack of its GEF. However, we have shown here that depletion of

RIN3, another GEF for Rab31, does not have the same effect, suggesting that the role of GAPex5 in Rab31-mediated EGFR trafficking may extend beyond its GEF activity. As GAPex5 has been shown to bind EGFR via Cbl, a second plausible explanation is that its presence in the EGFR-trafficking complex is essential for Rab31 function. It is likely that the presence of GAPex5 in the EGFR-containing complex is responsible for recruiting Rab31 onto EGFR-carrying endosomes. Once there, activated Rab31 can further engage its effector EEA1. We have not, however, been able at present to discern an interaction between Rab31 and other components of EGFR signalling such as Cbl (data not shown). Interestingly, we observed that depletion of GAPex5 resulted in dispersal of Rab31 from the TGN. This dispersal may impact the role of Rab31 in EGFR trafficking as well, possibly by disrupting the cycling of Rab31 between the TGN and the endocytic pathway (Rodriguez-Gabin et al., 2001).

GAPex5 is believed to be a GEF for Rab5 as well. Although it may seem somewhat counter-intuitive for a GEF to act as an activator for two Rab GTPases that we believe may act consecutively, examples can be found in other instances. For example, by engaging different subunits, the GEF TRAPP complex switches from acting on the cis-Golgi Rab Ypt1 (mediating ER-Golgi transport), to the trans-Golgi Rab Ypt32 (mediating exit from the Golgi) (Morozova et al., 2006). Alternatively, it is also possible that another GEF may be more critical for the Rab5-dependent steps. For example, it is suggested that RIN1 acts as a GEF with preferentially activity for Rab5a, the isoform that has been largely implicated in EGFR trafficking (Chen et al., 2009).

We also note that GAPex5 and RIN3, two different GEFs of Rab31, have different effects on the latter's localisation and modulation of EGFR trafficking. This highlights again that different GEFs play different roles in the activation and function

of a Rab, which may be dependent on its localisation or regulatory domains. In the case of Rab31-mediated trafficking of ligand-bound EGFR, GAPex5 appears to play a more important role.

Having seen that Rab31 plays a role in the trafficking of ligand-bound EGFR, we then went on to explore what physiological implications this may have. Our findings in this regard are presented in the following chapter.

6. Physiological role of Rab31 in the central nervous system

6.1 Chapter Introduction: Astrocytic cells and neurogenesis in the brain

In the embryonic mammalian brain, radial glia (RG), which are believed to derive from neuroepithelial cells, develop into the various progenitor cells that will eventually give rise to the three main cell types of the brain – the neurons, astrocytes and oligodendrocytes (Doetsch, 2003). During development, RG cells can be found in the germinal zone of the mouse telencephalon. Their cell soma resides in the embryonic germinal zone, while a single long process extends to the pial surface. They have both neuroepithelial and astroglial characteristics. For example, they are positive for both nestin and glial fibrillary acidic protein (GFAP), markers for progenitor and astrocytic cells respectively. RGs serve as neuronal and glial precursor cells as the embryonic brain develops (Gregg and Weiss, 2003). While RGs are not found in the adult brain, it is believed that as the animal matures, a population of RGs retain their neurogenic potential in the adult brain and become the neural progenitor cells of the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus, the two main areas of neurogenesis in the adult (Alvarez-Buylla et al., 2001). These nestin- and GFAP-positive cells (Imura et al., 2003) are known as Type B astrocytic cells in the SVZ and radial astrocytes in the SGZ, and are believed to be only a subset of the adult astrocytic population in this region (Kriegstein and Alvarez-Buylla, 2009; Duan et al., 2008). When examined by electron microscopy, these cells have the same ultrastructural characteristics as typical mature astrocytes, such as intermediate filament bundles, vascular end-feet, gap junction complexes and intercalating processes between other cell types (Ihrie and Alvarez-Buylla, 2008; Filippov et al., 2003). These cells are believed to give rise to new neurons, and possibly also the other glial

cell types (Liu et al., 2010) during adult neurogenesis. Type B cells, when activated, divide and give rise to Type C cells, which are immature precursors that eventually give rise to Type A cells, the migrating neuroblasts (García-Verdugo et al., 1998). These cells migrate to the olfactory bulb (in rodents) where they differentiate into interneurons (their exact target in humans is less well characterised). SGZ astrocytes, when stimulated, proliferate and give rise to progenitors (D cells) that mature into granule cells (G cells) that migrate to the inner layer and differentiate into hippocampal granule neurons (Seri et al., 2001) (Fig. 6.1).

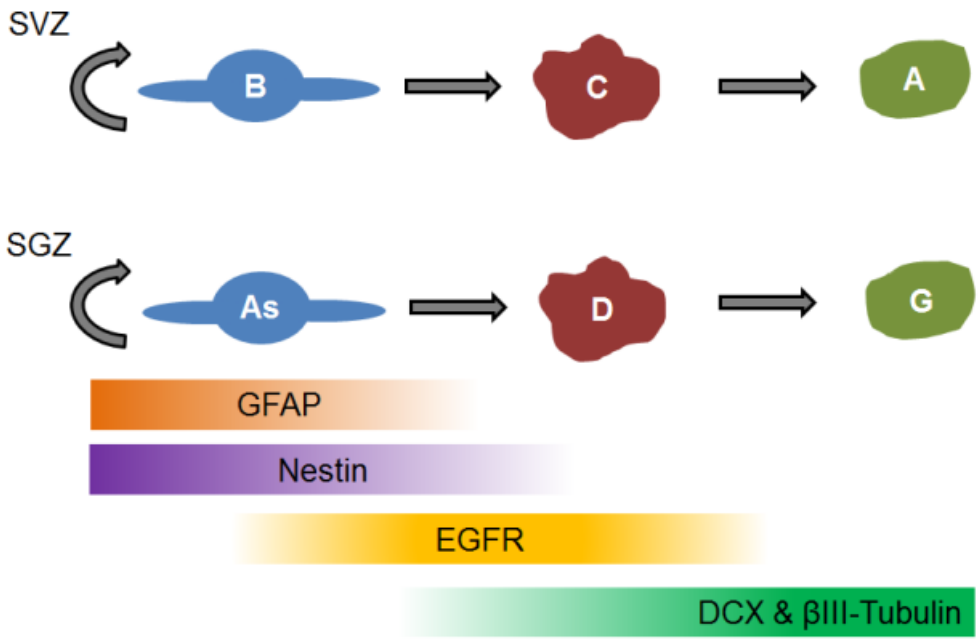


Fig. 6.1. Cells in the adult mouse neurogenic zones
Schematic of developmental progression of neural progenitor cells in the adult mouse brain and the expression of various markers. SVZ: Subventricular zone; SGZ: Subgranular zone. See text for details on Type A, B, C, D and G cells. As: SGZ astrocytic cells; GFAP: glial fibrillary acidic protein; EGFR: epidermal growth factor receptor; DCX: doublecortin.

Non-progenitor, mature astrocytes exist in the rest of the brain parenchyma. These parenchymal astrocytes, the characteristically star-shaped glial cells, are GFAP positive but lack nestin. When transplanted into neurogenic regions, they do not convert to progenitor cells (Ihrie and Alvarez-Buylla, 2008). They also express S100β, a calcium binding protein (Raponi et al., 2007). They are important for the functioning of the brain. Among other things, they provide metabolic support for neurons, maintain ion concentrations, support the blood-brain barrier and structure the brain. They are also important in neurogenesis. Co-cultures show that astrocytes stimulate neurogenesis from stem cells isolated from the SVZ and SGZ. This ability might be a regional characteristic of these astrocytes, as astrocytes isolated from the spinal cord do not have the same effect when co-cultured (Lim and Alvarez-Buylla, 1999; Song et al., 2002).

In our initial experiments detailing the expression profile of Rab31 in mouse tissues, we found Rab31 to be enriched in the brain (Ng et al., 2009). Immunohistochemical analysis revealed that Rab31 could be found in GFAP-positive cells in the adult mouse brain, suggesting that they are expressed in astrocytes. In the embryonic mouse brain, Rab31 was seen in nestin-positive cells, indicative of radial glia. In view of this, we reason that a closer look at a possible physiological role of Rab31 in neurogenic areas of the brain would be warranted.

6.2 Results: Rab31 in the adult rodent brain

The presence of Rab31 in radial glia, which serve as progenitor cells, prompted us to investigate whether Rab31 could also be found in neurogenic regions of the adult rodent brain. Indeed, immunostaining revealed Rab31-positive cells in both neurogenic regions of the mouse brain, namely the subventricular zone (SVZ)

and the subgranular zone (SGZ) of the hippocampal region which house neural progenitor cells (Fig. 6.2A). In a crude dissection of the rat brain to obtain tissue lysate from various subregions, we also found that Rab31 to be expressed at high levels in the hippocampus by Western blotting (Fig. 6.2B).

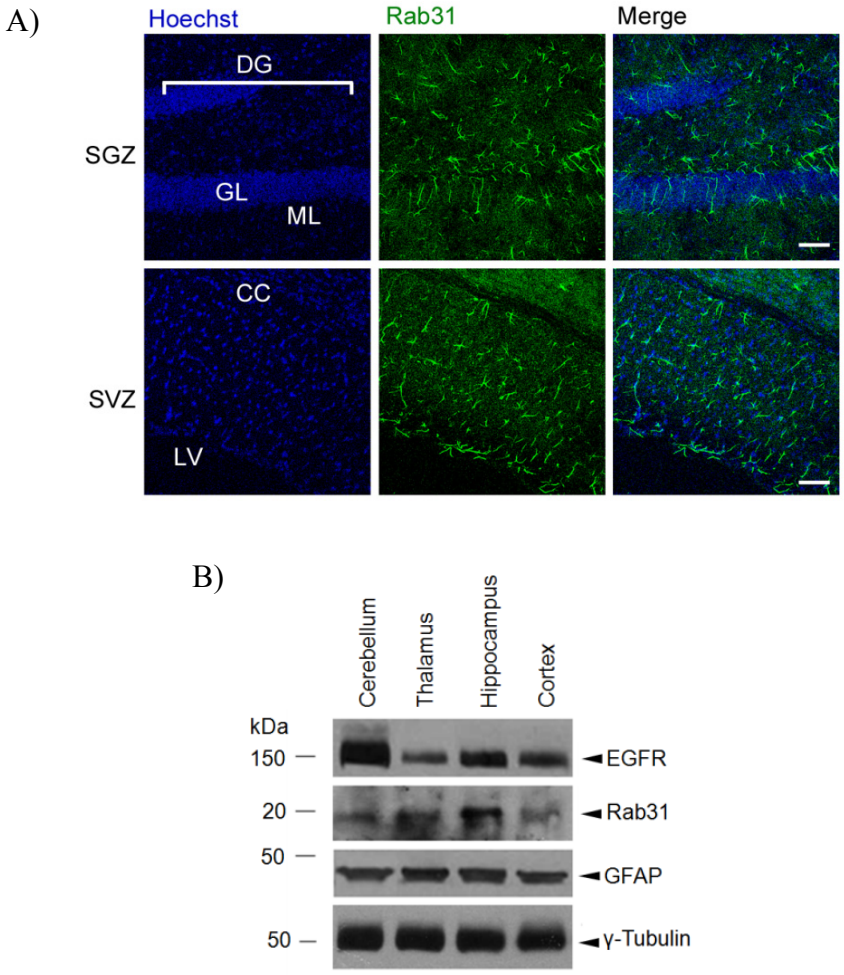


Fig. 6.2. Rab31 is found in the neurogenic zones of the adult rodent brain
A) Adult mouse brain was perfused with 4% paraformaldehyde, sectioned to 20 µm thickness on a cryostat and probed for Rab31 (green). SVZ and SGZ were identified by brain structural anatomy guided by the nuclear staining (Hoechst 33342, blue). In the upper panel, the SGZ lies along the top border of the GL. In the lower panel, the SVZ lies in the region between the LV and CC. DG: Dentate gyrus; GL: Granular layer; ML: Molecular layer; CC: Corpus callosum; LV: Lateral ventricle. Scale bar = 40 µm.
B) Various portions of the adult rat brain were dissected and lysed in extraction buffer. 100 µg of lysate was used for Western blot and probed for the various proteins indicated. Band densities were normalised against γ-tubulin.

Closer inspection of the hippocampal region using immunohistochemistry revealed that Rab31-positive cells could be found in both the dentate gyrus (DG) region and the CA1 region. These cells were also GFAP-positive, and were thus likely to be astrocytic in nature. Neural progenitor cells that are nestin and GFAP-positive are believed to reside in the subgranular zone (SGZ) of the DG, while astrocytes in the CA1 region are likely to be normal, mature parenchymal astrocytes (Raponi et al., 2007). While parenchymal astrocytes typically have the characteristic "star" shape, progenitor cells in the SGZ of the DG have a more radial-glial like appearance, with radial projections into the granular layer (Ihrie and Alvarez-Buylla, 2008). We observed that the Rab31-positive cells in the CA1 region were indeed of the typical star shape, while Rab31-positive cells in the DG could assume both astrocytic and radial glia morphologies (Fig. 6.3).

We thus took a closer look at the SGZ and attempted to characterise the nature of these Rab31-positive cells. The fibres of these Rab31-positive cells are found to extend from the SGZ into the granular layer, which is characteristic of the neuroprogenitor cells that reside there. These fibres were not TuJ positive (and are therefore not neurites) (Fig. 6.4A), but were positive for nestin, a neural progenitor marker (Fig. 6.4B). The morphology and marker phenotype suggests that these Rab31-positive cells were indeed neural progenitor cells. Perhaps most interestingly, these cells were also positive for EGFR (Fig. 6.4C). The presence of EGFR in the neurogenic zone has been documented by other groups, and dormant neural progenitor cells are believed to express a higher level of EGFR when they are activated (Alagappan et al., 2009; Pastrana et al., 2009). The presence of EGFR in GFAP-positive, Rab31-positive cells suggests that these might indeed be neural progenitor

cells, or the radial astrocytes, of the SGZ. It is likely that the Rab31-positive cells in the SVZ may be of a similar nature.

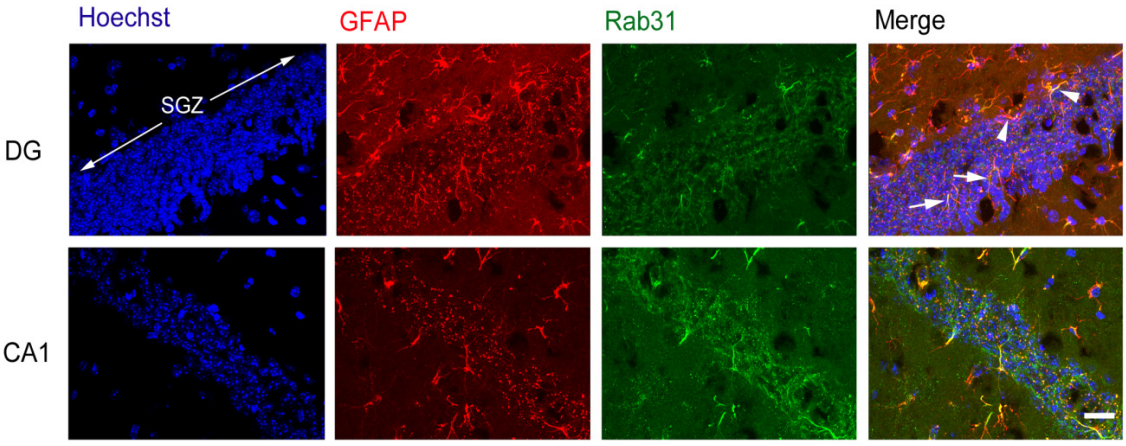


Fig. 6.3. Rab31 in the hippocampal region of the adult mouse brain
Adult mouse brain was perfused with 4% paraformaldehyde, sectioned to 20 µm thickness on a cryostat and probed with Rab31 (green) and GFAP (red) antibodies. DG and CA1 regions were identified by brain structural anatomy guided by the nuclear staining (Hoechst 33342, blue). The region of the SGZ is shown in the Hoechst-stained panel. Arrowheads indicate star-shaped cells typical of parenchymal astrocytes; arrows indicate radial astrocytes. Scale bar = 20 µm.

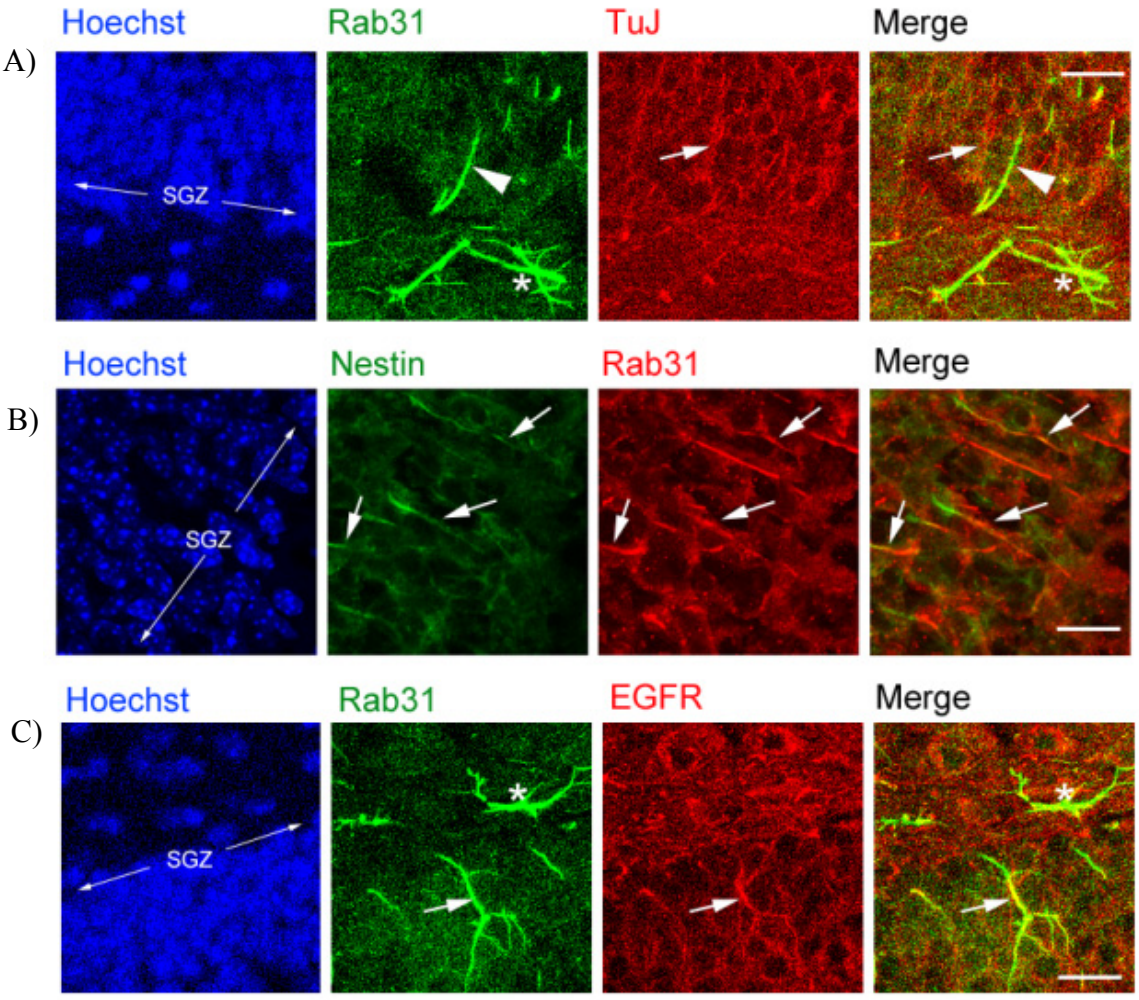


Fig. 6.4. Rab31-positive cells in the SGZ are not TuJ-positive but are nestin and EGFR-positive

Adult mouse brain was perfused with 4% paraformaldehyde, sectioned to 20 µm thickness on a cryostat and probed with Rab31 and various markers as indicated. Nuclei are marked by Hoechst 33342 (blue). The region of the SGZ is shown in the Hoechst-stained panel.

A) Arrowhead points to a Rab31-positive radial astrocyte (green) extending into the granular layer. Arrow points to a TuJ-positive cell (red). Asterisk indicates a parenchymal astrocyte.

B) Arrows indicate some of the Rab31 (red) and nestin-positive (green) fibres.

C) Arrow points to a Rab31 (green) and EGFR-positive (red) radial astrocyte.

Asterisk indicates a parenchymal astrocyte. Scale bar = 20 µm.

6.3 Results: Rab31 in neural progenitor cells

Rab31 in undifferentiated neural progenitor cells

Having seen that Rab31 could be found expressed in the neurogenic areas of the adult rodent brain, we asked what roles Rab31 might have in the neural progenitor cells. To investigate this, we used cultured neural progenitor cells (NPC) harvested from E15 mice, which would provide a platform for in vitro manipulation. These E15 NPCs are multipotent, EGF-responsive, and can be cultured for several passages as neurospheres. They are positive for nestin and PCNA, markers of radial glia and proliferating cells respectively. NPCs can also be induced to differentiate in various media that favour either the differentiation to astrocytes or neurons, respectively (Chojnacki and Weiss, 2008; Low et al., 2012).

In our cultured NPCs, Rab31 was found in the undifferentiated NPC at the perinuclear region (colocalising with the Golgi marker GM130) in most, if not all, cells. This Rab31-positive staining was obliterated when cells were transfected with Rab31 siRNA, showing that the staining is indeed specific for Rab31 (Fig. 6.5). These NPCs were also positive for both the progenitor cell-specific intermediate filament protein nestin (Fig. 6.6A) and proliferating cell nuclear antigen (PCNA) (Fig. 6.6B). These cells also appear to have moderate levels of EGFR (Fig. 6.6C). These latter characteristics are in accordance with that of typical undifferentiated NPCs.

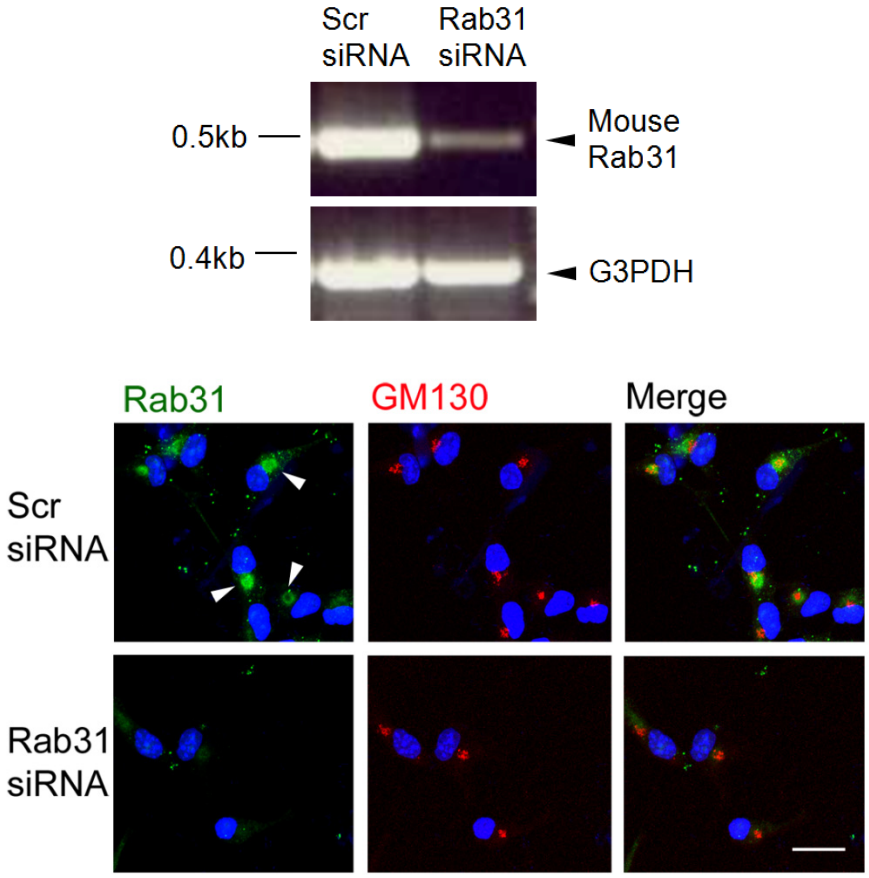


Fig. 6.5. Rab31 is expressed and localised to the perinuclear region in undifferentiated neural progenitor cells (NPC)
Mouse NPC were isolated from E15 mouse brain. Undifferentiated cells were plated, and transfected with scrambled (Scr) or Rab31 siRNA as indicated. Due to difficulty in picking up the Rab31 signal by Western blot of NPC lysates, depletion of Rab31 was quantified by RT-PCR after 48 h. Rab31 was 85% depleted as quantified by Image J. Cells were also fixed and probed for Rab31 (green) and GM130 (red). Arrowheads indicate examples of perinuclear Rab31 in the upper panel, which is not seen in the lower panel. Nuclei are visualised with Hoechst 33342 (blue). Scale bar = 20 μm.

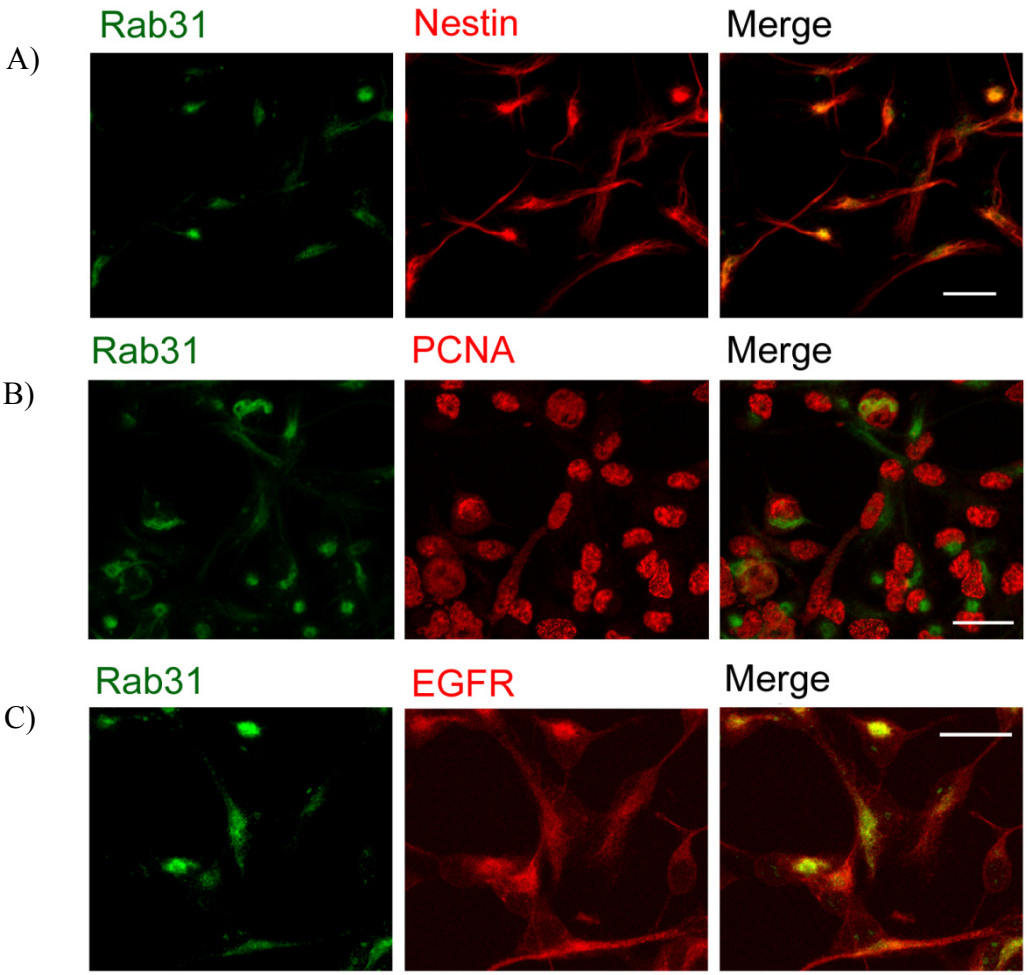


Fig. 6.6. Rab31- positive NPCs are positive for nestin, PCNA and EGFR
Mouse NPC were isolated from E15 mouse brain. Undifferentiated cells were plated, fixed and probed for Rab31 (green) and the various markers indicated (red). Scale bar = 20 μm.

Rab31 in differentiated neural progenitor cells

We next asked if Rab31 level changes, as the NPCs were induced to differentiate. Quantitative real-time PCR analysis of Rab31 mRNA transcripts as cells differentiated showed that Rab31 levels dropped transiently, but then increased, as NPCs differentiated to astrocytes, along with increasing GFAP levels and decreasing nestin levels. The changes seen were corroborated by immunofluorescence microscopy observations (Fig. 6.7). This observation therefore suggests that Rab31 expression may be linked to the differentiation process.

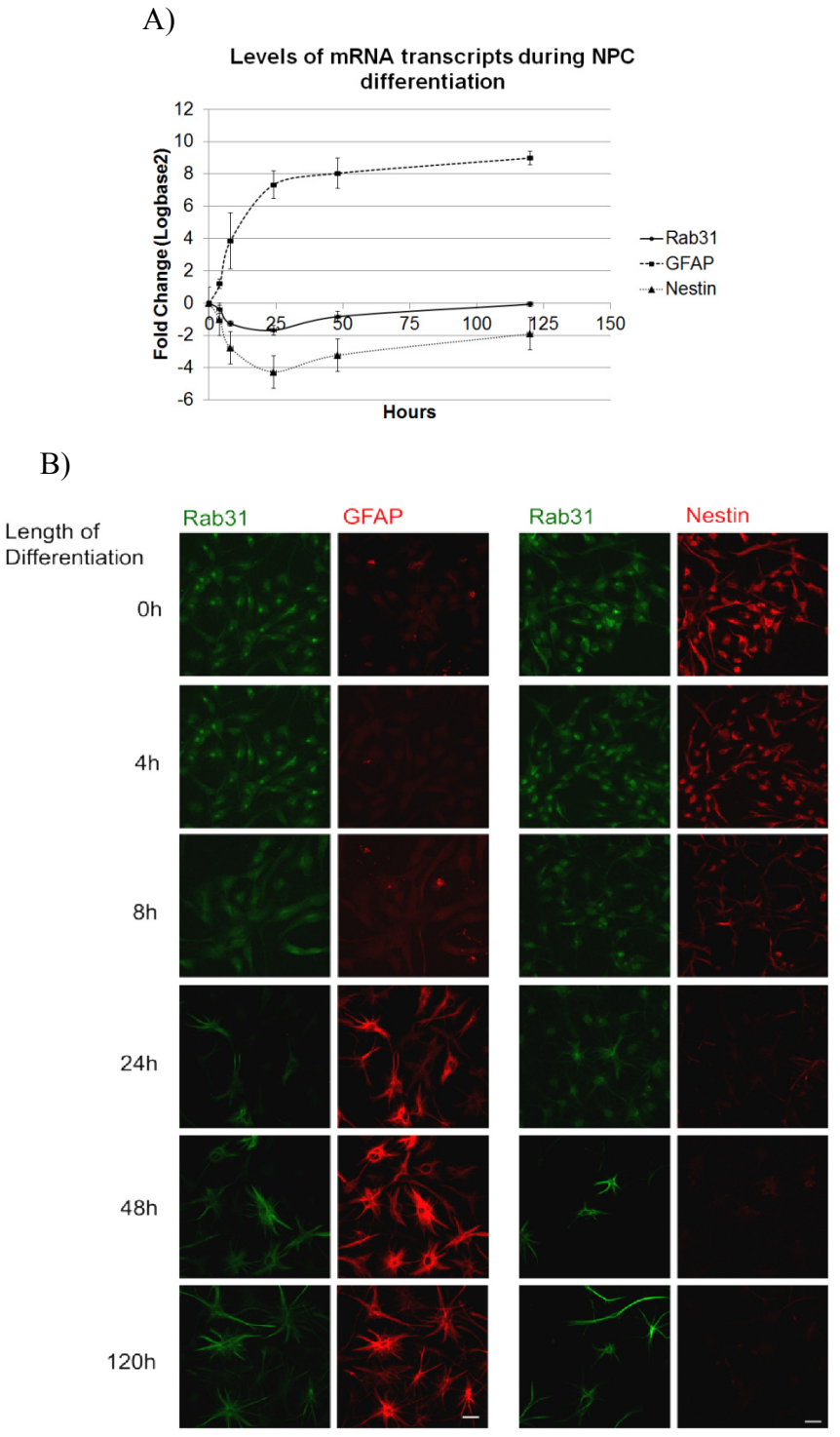


Fig. 6.7. Rab31 levels change in NPC induced to differentiate
A) Mouse NPCs were induced to differentiate to astrocytes. Total mRNA was harvested from cells at various time points indicated. Quantitative real-time PCR was used to determine the changes in mRNA levels of Rab31, GFAP, and nestin, normalised to Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) and represented as fold changes compared to the 0 h time point. Three independent experiments were performed and data is shown as mean ± SEM.
B) Cells were fixed at various times indicated and probed for Rab31 (green) and GFAP or nestin (red). Scale bar = 20 μm. h= hours.

In looking at how Rab31 levels change with differentiation, we observed that a subset of the GFAP-positive astrocytes obtained after differentiation were strongly immunopositive for Rab31. We thus took a closer look at this phenomenon. We allowed the NPCs to differentiate under 2 different conditions, one which promotes greater astrocytic differentiation and one which promotes greater neuronal differentiation. In both sets of differentiation conditions, we observed that Rab31 is found in elevated levels in a subset of the GFAP-positive population, but not in TuJ-positive cells examined (Fig. 6.8). The Rab31-positive cells also do not express 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), the marker for oligodendrocytes (Fig. 6.9A), or doublecortin (DCX), the marker for immature neurons (Fig. 6.9B). We also noted that these cells were now delocalised with cells positive for PCNA or EGFR (Fig. 6.10), indicating that these cells were no longer dividing progenitors. We quantified the number of GFAP-positive cells that highly express Rab31 and found that they constituted only a subset of the total GFAP-positive population (Fig. 6.11), as compared to in the undifferentiated NPC where the perinuclear Rab31 is seen in most, if not all, cells. These observations indicate that Rab31 is expressed in NPCs, but its expression is diminished as the NPC population differentiates. Its expression is then re-established when NPCs differentiate into astrocytes, but not when NPCs differentiate into neurons or other cell types. These observations are consistent with observations in vivo that Rab31 expression is most prominent in radial glia and adult astrocytes.

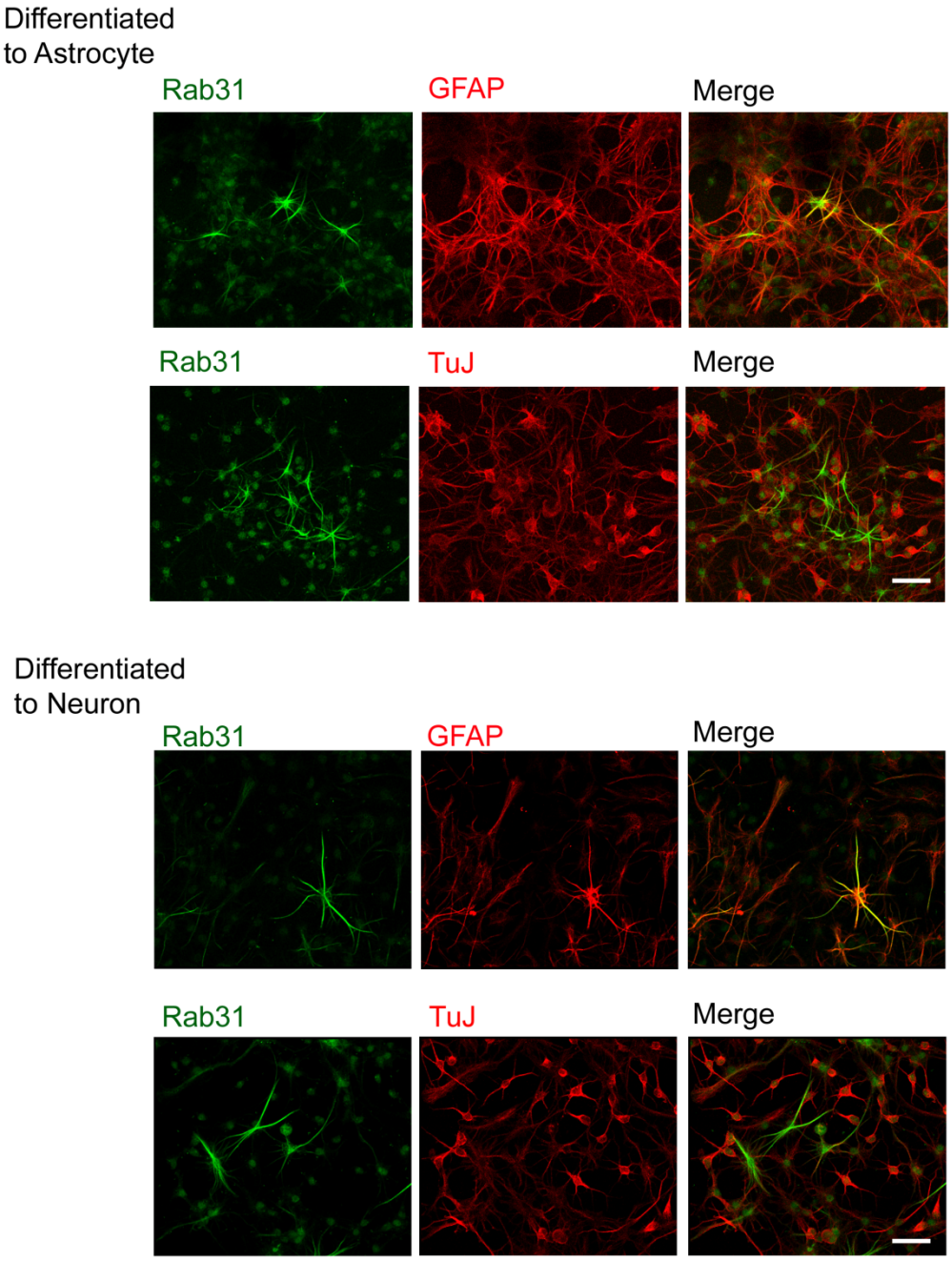


Fig. 6.8. Rab31 levels are elevated in a subset of GFAP-positive cells when NPCs are induced to differentiate
Mouse NPC were isolated from E15 mouse brain, plated and induced to differentiate under various conditions for 5 days, before fixation and immunofluorescence analysis. Cells were probed for Rab31 (green) and GFAP or TuJ (red). Rab31 is highly expressed in a subset of GFAP-positive cells but not TuJ positive cells, in both types of differentiation conditions. Scale bar = 50 μm.

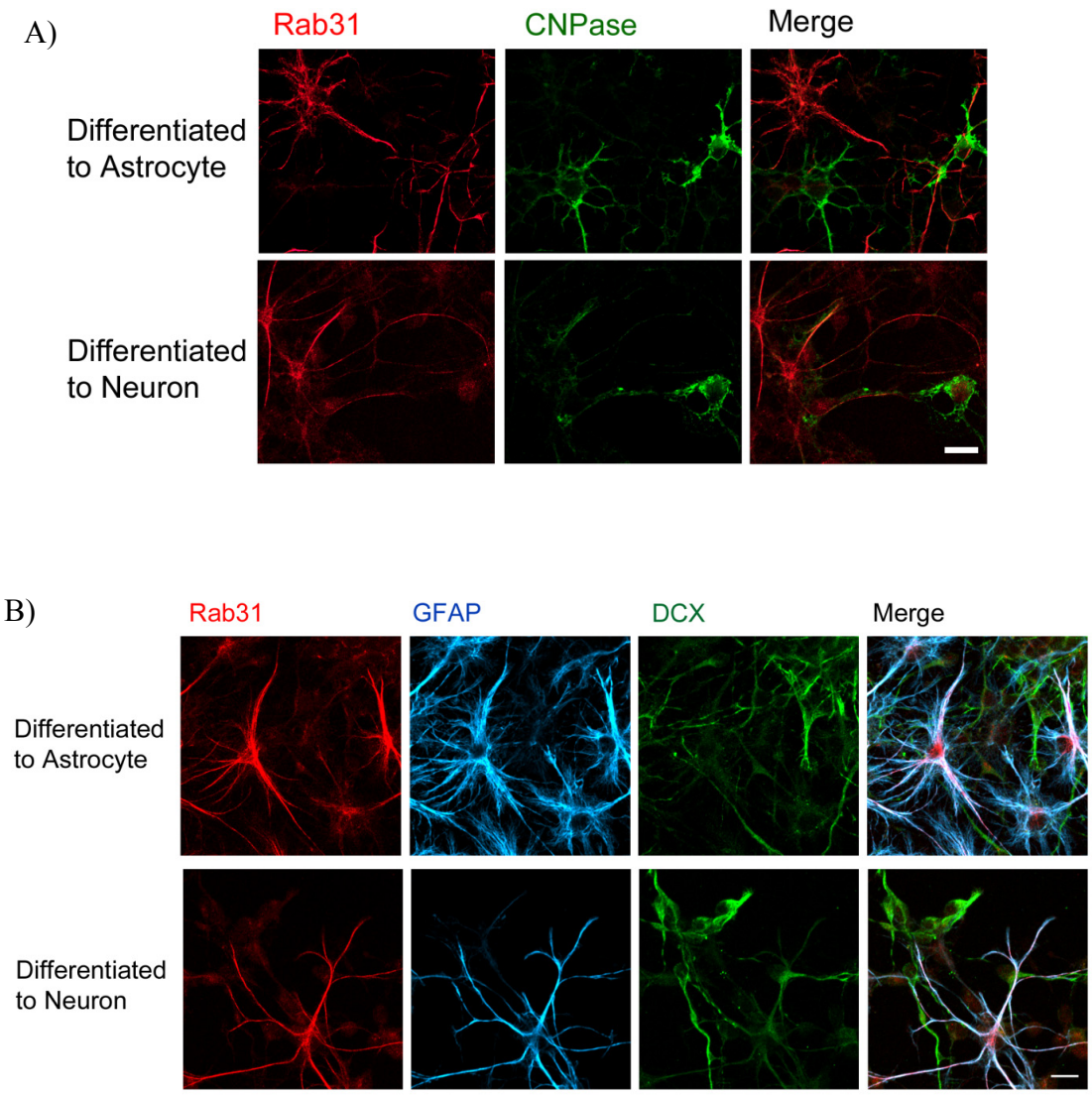


Fig. 6.9. Elevated Rab31 levels are not found in CNPase and DCX-positive cells in NPC induced to differentiate
A) Mouse NPC were isolated from E15 mouse brain, plated and induced to differentiate under various conditions for 5 days, before fixation and immunofluorescence analysis. Cells were probed for Rab31 (red) and CNPase (green). Scale bar = 20 µm.
B) Mouse NPCs were isolated from E15 mouse brain, plated and induced to differentiate for 5 days, before fixation and immunofluorescence analysis. Cells were probed for Rab31 (red), GFAP (pseudo-coloured blue) and DCX (green). Scale bar = 20 µm.

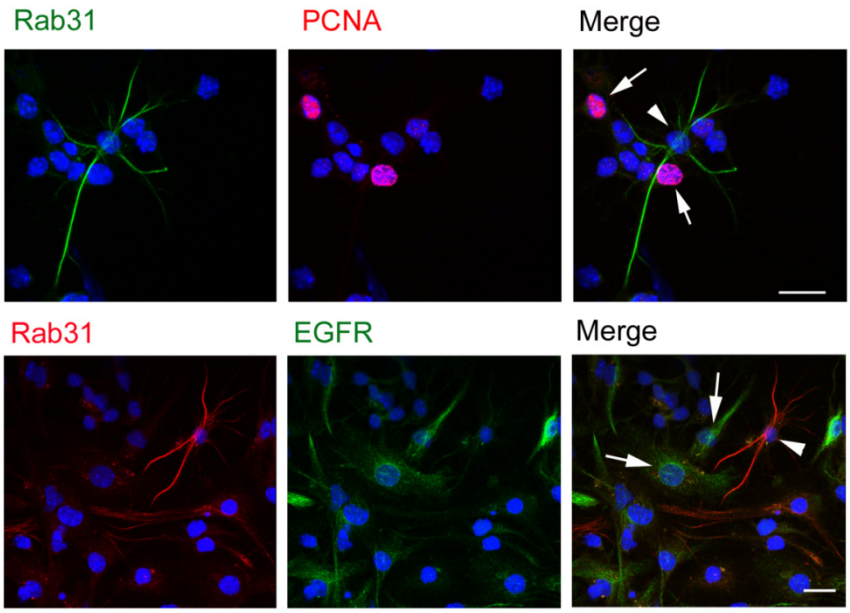


Fig. 6.10. Cells with elevated Rab31 levels are delocalised from cells that are still PCNA and EGFR-positive in NPC induced to differentiate
Mouse NPCs were isolated from E15 mouse brain. Undifferentiated cells were plated and induced to differentiate. Cells were fixed and probed for Rab31 and various markers as indicated. Arrows indicate PCNA or EGFR positive cells; arrowhead points to a Rab31-positive cell. Nuclei were visualised with Hoechst 33342 (blue). Scale bar = 20 µm.

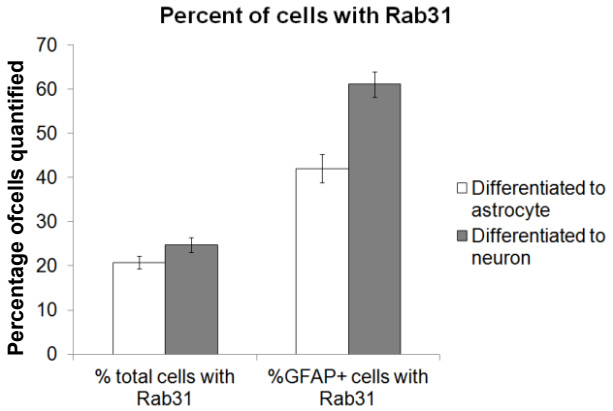


Fig. 6.11. Percentage of cells with elevated levels of Rab31 in NPC induced to differentiate
Mouse NPCs were isolated from E15 mouse brain. Undifferentiated cells were plated, and induced to differentiate. Cells were fixed and probed for Rab31 and GFAP. Number of Rab31-positive cells was quantified and presented graphically as a percentage of total cells and total GFAP-positive cells counted, respectively. 38 cells in 3 independent experiments were analysed and data is shown as mean ± SEM.

Effect of Rab31 depletion and overexpression on differentiation of neural progenitor cells

From the findings above, we can perhaps postulate 2 potential roles for Rab31: a) Given the ubiquitous presence of Rab31 in undifferentiated NPC and its initial diminishment as cells differentiate, Rab31 may help to maintain NPCs in an undifferentiated form; b) The subsequent increase in Rab31 levels in a subset of astrocytic cells suggests that Rab31 may also be a cell fate determinant for the differentiation of NPCs to astrocytes versus neurons, and perhaps also the subtype of astrocytes that emerges at the end.

To investigate the first possibility, we silenced Rab31 expression using GFP-tagged Rab31 shRNA and enhanced the rate of shRNA introduction into the NPCs by using retroviral transduction, and maintained these cells in EGF- and fibroblast growth factor 2 (FGF-2)-supplemented culture (Fig. 6.12). After 48 h, we investigated if there was extraneous differentiation observed in NPCs in which Rab31 was depleted, and found no morphological evidence that loss of Rab31 would itself induce cells to differentiate, as assessed by staining with nestin (Fig. 6.13A). This was corroborated by a Western blot which showed no decrease in nestin and PCNA levels (Fig. 6.13B). We also investigated if the converse treatment, i.e. Rab31 overexpression (Rab31 OE), would affect the undifferentiated state of NPCs. Transfection with Myc-Rab31 did not affect the NPCs in an undifferentiated state, which remained nestin-positive (Fig. 6.14). Together, the results suggest that manipulation of the levels of Rab31 does not have a significant impact on the undifferentiated state of NPCs.

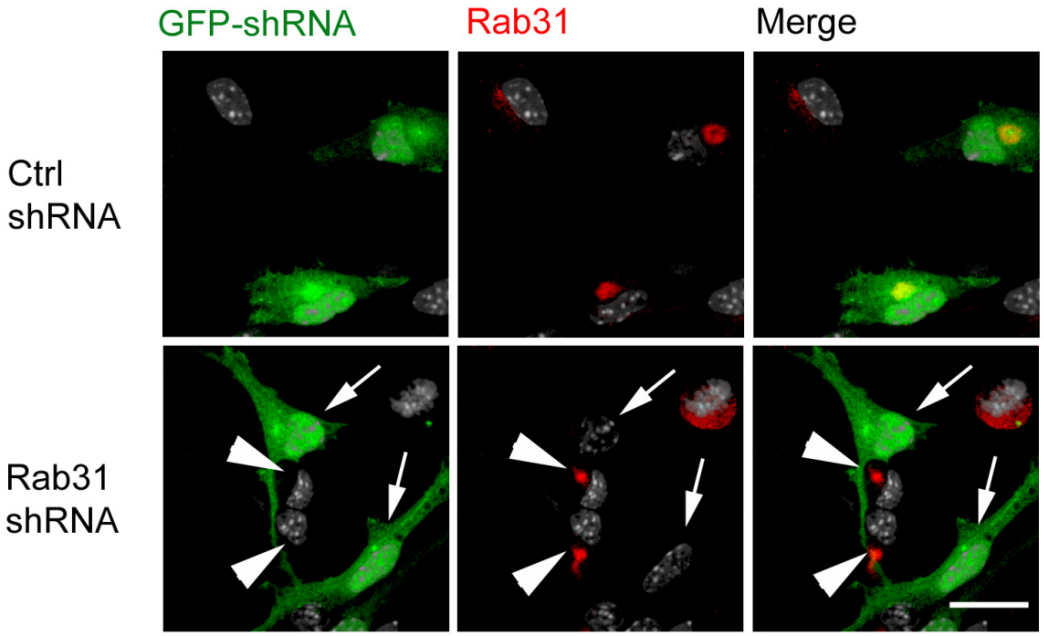


Fig. 6.12. Depletion of Rab31 by GFP-tagged shRNA retroviral transduction
Mouse NPCs were isolated from E15 mouse brain. Undifferentiated cells were plated and cultured with medium containing control (Ctrl) or GFP-Rab31 shRNA retroviruses. After 48 h, cells were fixed and immunofluorescence staining was performed to assess extent of Rab31 depletion. In the Rab31 shRNA panel, arrows indicate GFP-positive cells (green), where Rab31 (red) staining is not present, in contrast with arrowheads which indicate non-transduced cells where Rab31 staining persists. Scale bar = 20 μm.

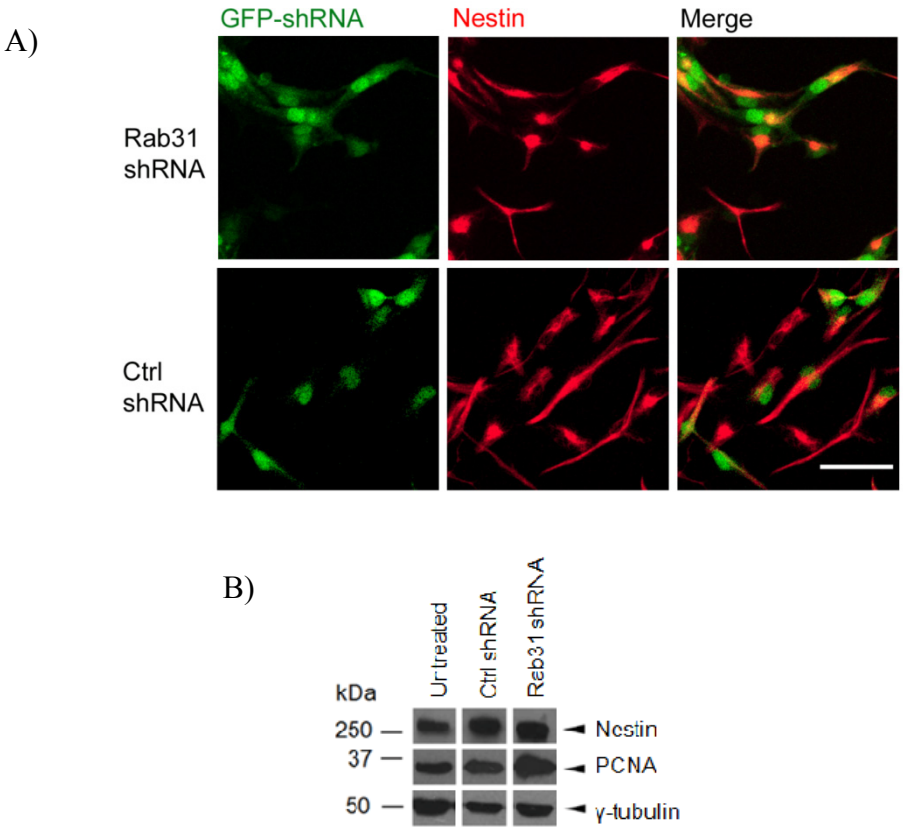


Fig. 6.13. Depletion of Rab31 does not affect undifferentiated state in NPCs
Mouse NPCs were isolated from E15 mouse brain. Undifferentiated cells were plated and cultured with medium containing Ctrl or GFP-Rab31 shRNA retroviruses.
A) After 48 h, cells were fixed and immunofluorescence staining for nestin (red) was performed. Scale bar = 20 μm.
B) Cells were also harvested for cell lysate and analysed by Western blot for nestin, PCNA, and γ-tubulin.

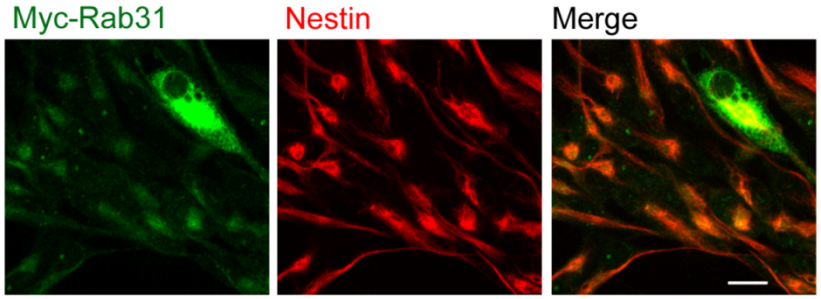
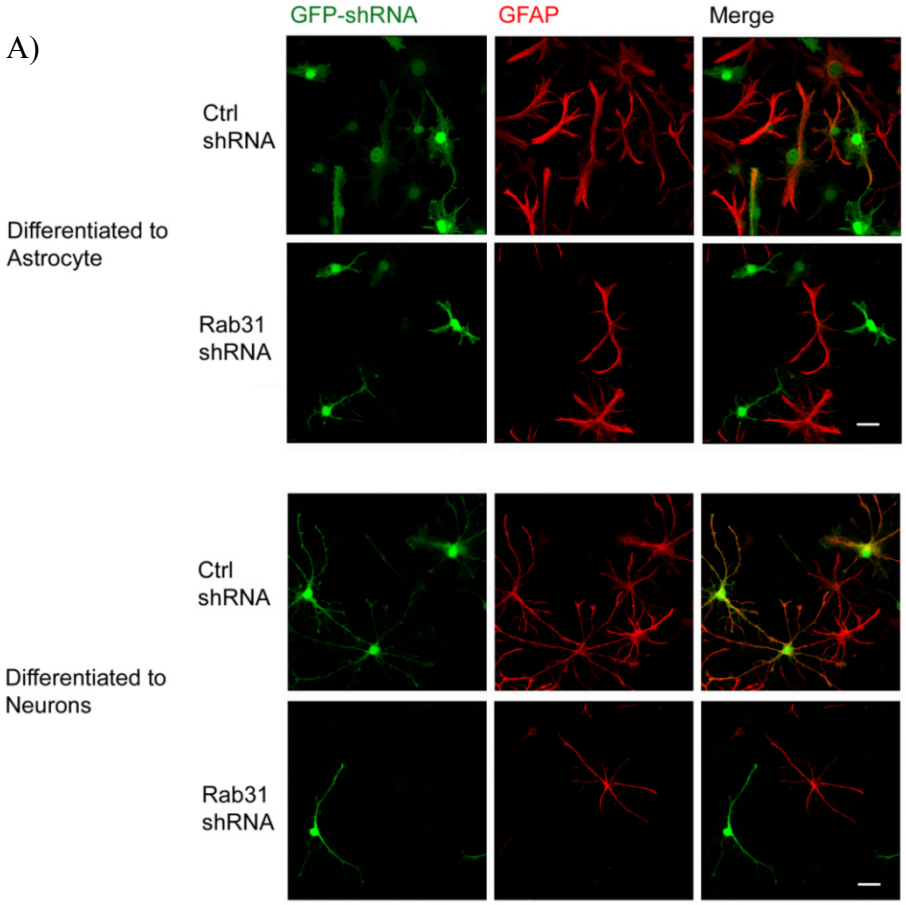


Fig. 6.14. Myc-Rab31 overexpression did not spontaneously induce NPCs to differentiate
Mouse NPCs were isolated from E15 mouse brain. Undifferentiated cells were plated and transfected with Myc-Rab31. After 48 h, cells were fixed and probed for Rab31 (green) and nestin (red). Scale bar = 20 μm.

We next investigated whether Rab31 played a deterministic role in the differentiation of NPCs. In conditions suited for both astrocyte and neuronal differentiation, there were significantly fewer GFAP-positive cells obtained when Rab31 was silenced (as indicated by the presence of GFP), compared to controls (Fig. 6.15A). We quantified this and found that in cells with Rab31 depletion there was a reduced percentage of GFAP-positive cells obtained (Fig. 6.15B). There was no difference in the percentage of DCX-positive cells (Fig. 6.16). We then overexpressed Rab31 and induced the NPC to differentiate (Fig. 6.17). (As there was little observable difference between cultures in which astrocyte or neuronal-favouring differentiation medium was used, subsequent data shown is from experiments performed with astrocyte-favouring differentiation medium). Overexpression of Rab31 (Rab31 OE) enhanced the percentage of GFAP-positive astrocytes. Together, the results suggest that depletion of Rab31 reduces, while overexpression of Rab31 enhances, the percentage of GFAP-positive astrocytes obtained when NPCs are induced to differentiate (Fig. 6.18). It is therefore likely that Rab31 is important for the differentiation of NPCs along the astroglial lineage.

A)


GFP-shRNA
GFAP
Merge
Ctrl shRNA
Differentiated to Astrocyte
Rab31 shRNA
Ctrl shRNA
Differentiated to Neurons
Rab31 shRNA

B)

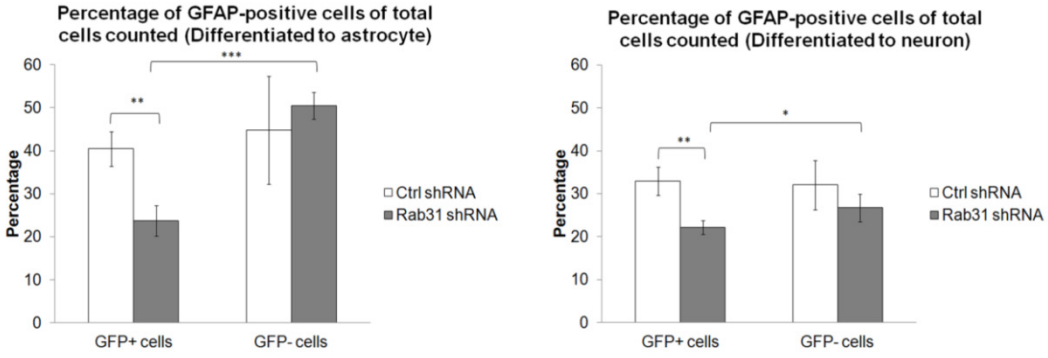

Percentage of GFAP-positive cells of total cells counted (Differentiated to astrocyte)
Percentage
60
50
40
30
20
10
0
**
***
GFP+ cells
GFP- cells
Ctrl shRNA
Rab31 shRNA
Percentage of GFAP-positive cells of total cells counted (Differentiated to neuron)
Percentage
60
50
40
30
20
10
0
**
*
GFP+ cells
GFP- cells
Ctrl shRNA
Rab31 shRNA

Fig. 6.15. Depletion of Rab31 by GFP-tagged shRNA retroviral transduction reduced the number of GFAP-positive cells obtained when NPCs were induced to differentiate
Mouse NPCs were isolated from E15 mouse brain. Undifferentiated cells were plated and cultured with medium containing Ctrl or GFP-Rab31 shRNA retroviruses. After 48 h, cells were induced to differentiate as indicated.
A) After 5 days cells were fixed and immunostained for GFAP (red). Cells that have taken up the retroviral shRNA express GFP (green). Scale bar = 20 μm.
B) Number of GFAP-positive cells was quantified and presented graphically as a percentage of total cells counted. Left panel is obtained from cells cultured in medium favouring astrocyte differentiation while right panel is obtained from that favouring neuronal differentiation. White bars represent counts from cells cultured with medium containing the control shRNA retroviruses (Ctrl) while shaded bars represent counts from cells cultured with Rab31 shRNA retroviruses. 35 cells in 3 independent experiments were analysed and data is shown as mean ± SEM. *P<0.05, **P<0.01 and ***P<0.001 as determined by one-way ANOVA.

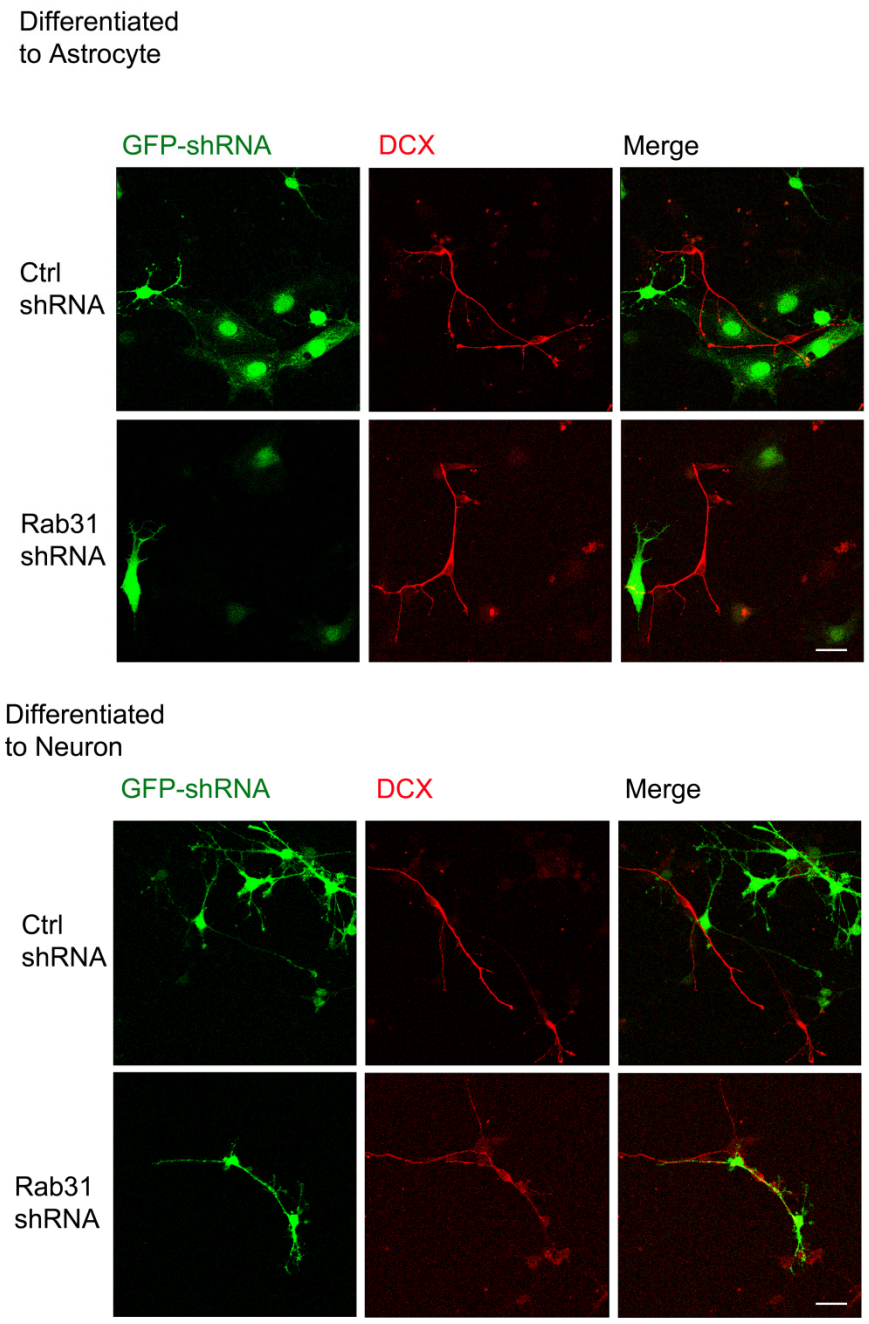


Fig. 6.16. Depletion of Rab31 by GFP-tagged shRNA retroviral transduction did not affect the percentage of DCX-positive cells obtained when NPCs were induced to differentiate

Mouse NPCs were isolated from E15 mouse brain. Undifferentiated cells were plated and cultured with medium containing Ctrl or GFP-Rab31 shRNA retroviruses. After 48 h, cells were induced to differentiate as indicated. After 5 days cells were fixed and immunostained for DCX (red). Cells that have taken up the retroviral shRNA express GFP (green). Scale bar = 20 μm.

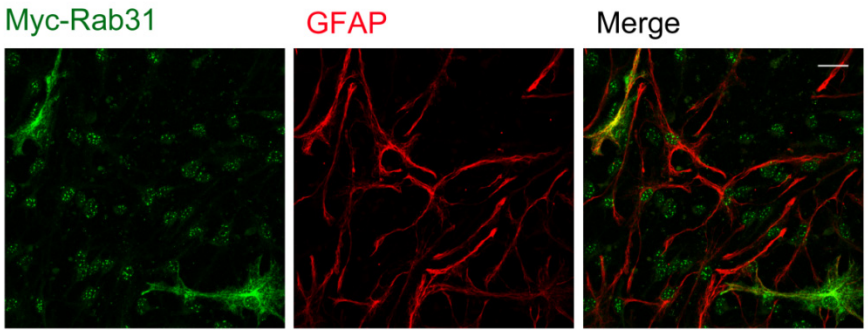


Fig. 6.17. Cells overexpressing Myc-Rab31 also express GFAP when NPCs were induced to differentiate
Mouse NPCs were isolated from E15 mouse brain. Undifferentiated cells were plated and transfected with Myc-Rab31. After 48 hours, cells were induced to differentiate. After 5 days cells were fixed and immunofluorescence staining for Rab31 (green) and GFAP (red) was performed. Scale bar = 20 μm.

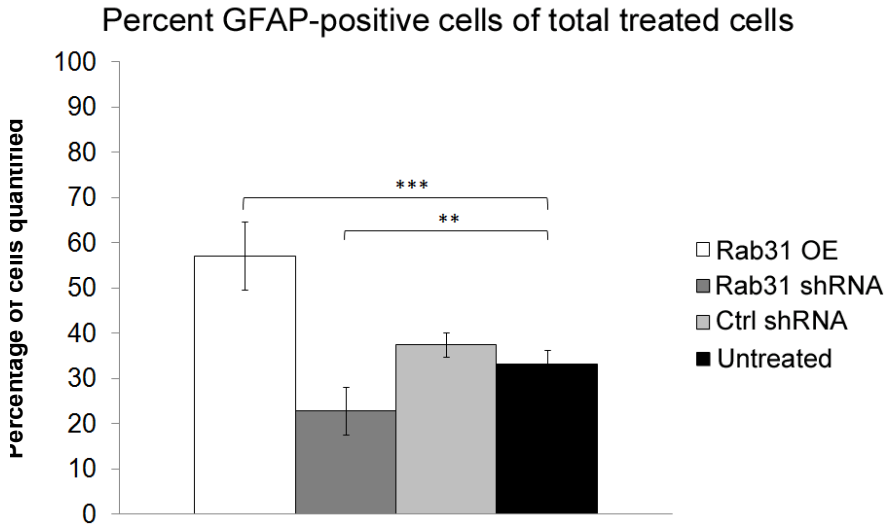


Fig. 6.18. Effect of manipulation of Rab31 levels on differentiation of NPCs
NPCs were treated as indicated and induced to differentiate after 48 h. Cells were maintained in culture for 5 days before fixing and immunofluorescence staining for GFAP. Number of GFAP-positive cells was quantified and presented graphically as a percentage of total treated cells counted. Treated cells were identified by the Myc-Rab31 expression (for Rab31 OE) or the GFP expression (for shRNA-treated cells). 42 cells in 3 independent experiments were analysed and data is shown as mean ± SEM. **P<0.01 and ***P<0.001 as determined by one-way ANOVA.

Effect of EGF withdrawal on differentiation of neural progenitor cells

As presented and discussed in Chapters 4 and 5, Rab31 plays a role in the trafficking of ligand-bound EGFR. Depletion of Rab31 delayed the degradation of EGFR, by hindering its entry into late endosomes, while overexpression of Rab31 enhanced the degradation (Fig. 4.8B). It is conceivable that the effect of Rab31 on the differentiation of NPCs was due to its role in EGFR trafficking. Increased levels of Rab31 increase the degradation of EGFR, thus decreasing the time which ligand-bound EGFR is able to signal. This could in turn affect the differentiation programme when cells were induced to differentiate, as EGFR signalling plays an important role in the proliferation and differentiation of progenitor cells. We postulated that a withdrawal of EGF from the media in which NPCs were cultured may crudely mimic the situation in which Rab31 was overexpressed in these cells. However, when we first withdrew EGF from the media in which the undifferentiated NPCs were cultured, and then induced the NPC to differentiate, we found that the number of GFAP-positive astrocytes obtained was reduced (Fig. 6.19A). We quantified this and found that EGF withdrawal from the media for undifferentiated NPC subsequently reduced the percentage of GFAP-positive cells obtained when the NPC were induced to differentiate (Fig. 6.19B), rather than increased in the case of a Rab31 overexpression. The possible reasons for this finding are discussed in the following section.

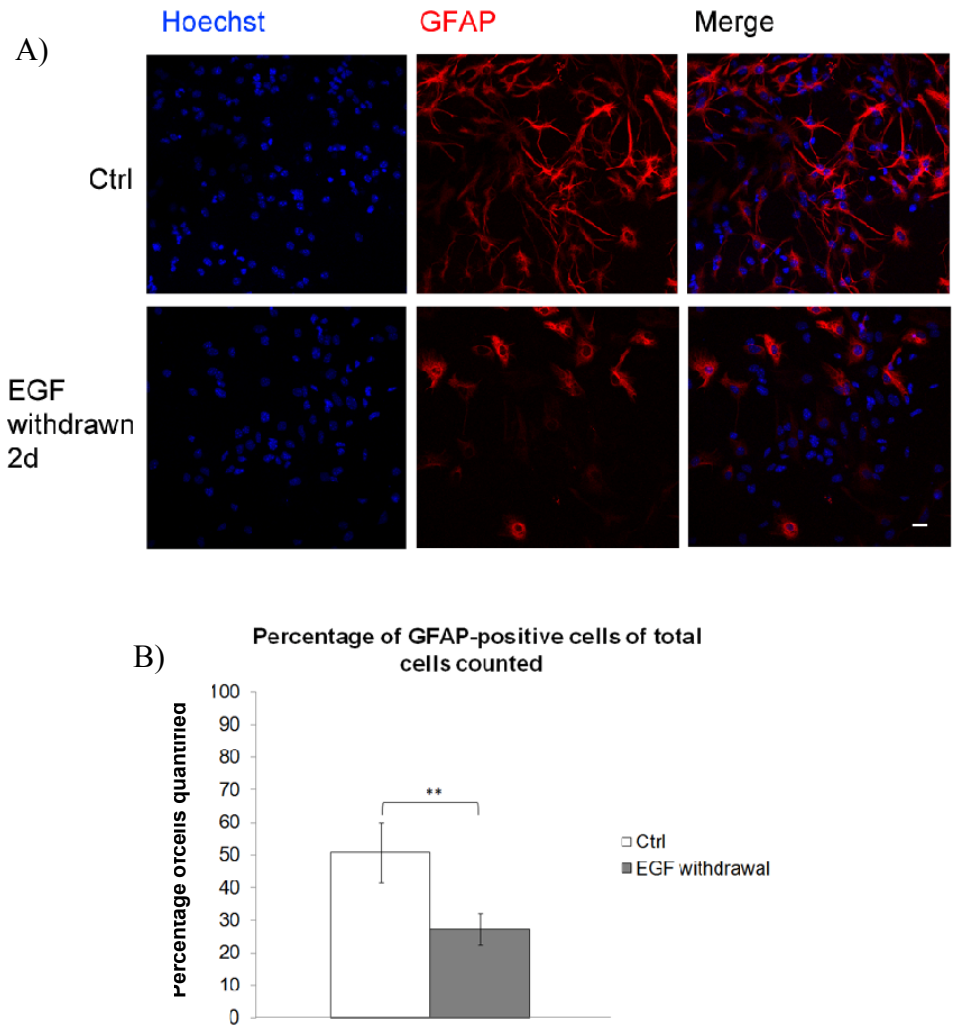


Fig. 6.19. Withdrawal of EGF from NPC culture media before differentiation reduced the number of astrocytes obtained

Mouse NPCs were isolated from E15 mouse brain. Undifferentiated cells were plated and EGF was withdrawn from the culture med ia. After 48 hours, cells were induced to differentiate.

A) After 5 days cells were fixed and immunofluorescence staining for GFAP (red) was performed. Nuclei were visualised with Hoechst 33342. Scale bar = 20 μm.

B) Number of GFAP-positive cells was quantified and presented graphically as a percentage of total cells counted. 32 cells in 3 independent experiments were analysed and data is shown as mean ± SEM. **P<0.01 by Student's T-test.

6.4 Chapter Discussion: Possible role of Rab31 in NPCs and astrocytes

Role of Rab31 in NPCs

Our results suggest that Rab31 exerts a positive influence on the generation of astrocytes from neural progenitors, and has therefore a distinct physiological role to play in the mammalian brain. At present, the mechanism by which Rab31 exerts its effect is not known. We could postulate that it may be linked to the role of Rab31 in EGFR trafficking as shown in A431 and HeLa cells, in which Rab31 depletion reduced, while Rab31 overexpression enhanced, the movement of EGFR into late endosomes, thus affecting its eventual degradation in lysosomes.

EGFR signalling is important to the survival and function of NPCs. The amount and effect of EGFR signalling varies during different stages of development. At early stages, a low level of EGFR signalling was shown to be required for proliferation of neural progenitor cells (Liu and Neufeld, 2007). Overexpression of EGFR (via retroviral transduction in vitro and in vivo) at this stage causes NPCs to present the characteristics of differentiating astrocytes at later stages (Burrows et al., 1997). Our observation of the presence of Rab31 in NPCs (Fig. 6.6) may thus reflect their role in a fast turnover of ligand-bound EGFR, keeping EGFR signalling at a low level required for the proliferation of neural progenitor cells. Because of the elongated nature of NPCs and the lack of antibodies suited for immunocytochemical observation of endocytic trafficking in this cell type, we were unable, at this point, to fully explore the dynamic nature of Rab31-mediated EGFR trafficking in NPCs. Although in our hands manipulating Rab31 levels did not affect the undifferentiated state of NPCs, this may simply be a result of other feedback or redundancy mechanisms in place.

At later stages of embryonic development, high level of EGFR signalling is required for differentiation to astrocytes (Liu and Neufeld, 2007). It has been shown that

EGFR and its ligands gradually increase in the developing CNS, coincident with gliogenesis (Tropepe et al., 1999; Kornblum et al., 1997). Furthermore, during asymmetrical division of progenitor cells, daughter cells with high EGFR levels become astrocytes while those with low levels become oligodendrocytes (Sun et al., 2005). In our observations, when NPCs are induced to differentiate, Rab31 levels show a dip initially (Fig. 6.8). This may aid in reducing the rate of degradation of ligand-bound EGFR, prolonging the effect of its signalling, to enable the differentiation to astrocytes.

A role in regulating the duration of EGFR signalling, however, cannot properly explain why Rab31 depletion would decrease (rather than increase) the eventual number of astrocytes obtained, and vice versa. One possible reason could be linked to a recent observation that EGF signalling, while enhancing the progression of radial glia to immature astrocytes (which are both nestin- and GFAP-positive), subsequently inhibits the progression from immature astrocytes to mature astrocytes (which express S100β along with GFAP) (Raponi et al., 2007). The increase in EGFR signalling caused by our Rab31 depletion studies might have resulted in a hindrance to the complete development of the astrocytes in culture, thus resulting in a decreased percentage of astrocytes that survive in culture.

Although we attempted to further explore the idea of a Rab31-EGFR effect on astrocytic differentiation by withdrawing EGF from the culture medium, we were unable to show a similar effect to Rab31 overexpression. One possible explanation for this is that EGF withdrawal is a poor mimic of the effect of overexpression of Rab31. Firstly, although EGF is removed from the growth media, the FBS used in the differentiation media also contains an undefined amount of growth factors. Secondly, overexpression of Rab31 certainly would not completely abrogate EGFR signalling,

but rather alters its strength and/or duration. Thirdly, manipulation of Rab31 levels may alter subtle balances in trafficking or trigger feedback mechanisms that affect EGFR signalling or trafficking in unexpected ways. We should also note that besides EGF, a myriad of other factors are involved in signalling in NPCs, including FGF-2, platelet derived growth factor (PDGF) and TGFα (Jackson et al., 2006). While we have found that Rab31 does not participate in the trafficking of FGF receptor and PDGF receptor (data not shown), TGFα also binds to EGFR and its signalling may therefore also be affected by Rab31.

Ultimately, it is the specific combination of ligand and receptor levels, at different stages of embryonic development, which determine the response of neural progenitor cells. To gain a better understanding of how Rab31 affects this process, we would need to manipulate Rab31 levels at various stages in embryonic development *in vivo*. Conceivably, manipulating Rab31 levels (and hence EGFR signalling) during the stage at which proliferation of NPCs predominates would differ from stages at which neurons are being generated, which in turn would differ from the early postnatal stages at which more glial cells are being generated.

In the adult mouse brain, NPCs are generally quiescent until activated, at which time they upregulate EGFR and become EGF responsive (Pastrana et al., 2009; Alagappan et al., 2009). This results in enhanced proliferation and migration capabilities, and reduced differentiation (Ayuso-Sacido et al., 2010). The reason for the high levels of Rab31 observed in these quiescent NPCs in our adult mouse brain cryosection is unclear, given that the quiescent cells are not EGF-responsive. It is possible that Rab31 has a role separate from that of EGFR trafficking in these cells, or that they regulate a low, constitutive level of EGFR signalling present in these cells. It might even be that these cells reflect a small population that are activated NPCs. It

would be interesting to observe how Rab31 levels in the neurogenic zones of the adult mouse brain might change during CNS injury, and whether manipulation of their levels would affect the response of the quiescent NPCs to injury.

Role of Rab31 in astrocytes

We have also observed Rab31 in astrocytes in the adult mouse brain and in a subset of GFAP-positive cells in our NPC culture induced to differentiate. EGFR signalling induces astrocytes to provide a permissive environment for neurite outgrowth in the developing CNS (Liu and Neufeld, 2004). In the adult CNS, EGFR is absent from mature astrocytes (Gómez-Pinilla et al., 1988) but reappears together with nestin (Herrmann and Aebi, 2000) in reactive astrocytes in response to injury, which changes their phenotypic characteristics (Liu and Neufeld, 2007; Liu, 2006). Notably, these astrocytes are distinct from those believed to be neural progenitors, as they also express S100β, which is a distinct marker for mature astrocytes (Cho et al., 2013; Ihrie and Alvarez-Buylla, 2008). At present the reason for the high levels of Rab31 seen in a subset of astrocytes in our adult mouse brain cryosection and differentiated NPC culture is not known. Given that EGFR signalling is very much reduced in mature astrocytes, the enrichment of Rab31 in a subset of the astrocytic population, even those outside of the neurogenic regions, may be reflective of a separate role of Rab31 from EGFR trafficking. Rabs, for example, are believed to play a role in the exocytosis of neuropeptides or the recycling of transporters, such as the glutamate-aspartate transporter, on astrocytic membranes (Kreft et al., 2009). Rab31 may play an as-yet-unknown role in these areas. It would also be interesting to see how Rab31 levels change in parenchymal astrocytes in the injured CNS, when the astrocytes become EGF-responsive.

7. Conclusion and future perspectives

7.1 General conclusions

In this thesis, Chapter 3 describes attempts in exploring some of the possible mechanisms by which Rab31 is faithfully localised to the trans-Golgi network, and we show that it is a combination of functional domains and interacting proteins that mediate this process. Interestingly, we found that two different GEFs of Rab31, GAPex5 and RIN3, had different influences on Rab31 localisation. We also observed that Rab31 could also be localised, in part, to the endosomal network. Given that other members of the Rab5 subfamily (to which Rab31 belongs) participate in endocytic trafficking steps, including those of cell surface receptors such as EGFR, we sought to investigate the participation of Rab31 in the endocytic trafficking of EGFR. We show in Chapter 4 and 5 that Rab31 participates in the transition of ligand-bound, internalised EGFR from the early to late endosome, by engaging in a trafficking complex that includes EGFR and EEA1.

These results are intriguing for several reasons. Firstly, it suggests that although Rab31 is largely and primarily localised to the TGN, there is a fraction found on what is likely to be endosomes, and this endosomal pool of Rab31 might possibly play a physiological role in the cell. Some groups have suggested that Rab31 moves from the TGN onto tubulovesicular structures and is responsible for anterograde transport between the TGN and endosomes (Rodriguez-Gabin et al., 2001). Here, we provide evidence that Rab31 also plays a role in retrograde transport in the endocytic pathway. Secondly, Rab31 appears to be part of a larger trafficking complex that includes both EGFR and EEA1. Previously, Rab25 and Rab21 were the only Rabs thus far that have been shown to interact directly with cell surface receptors, namely integrin (Mai et al., 2011) and more recently, EGFR (Yang et al., 2012). Thirdly,

our results indicate that Rab31 is a player in the endocytic trafficking pathway of EGFR. We postulate that the formation of Rab31-EGFR trafficking complex, together with EEA1, is subsequently essential for the entry of the ligand-bound receptor into the late endosomes. This is perhaps similar to the role that has been recently postulated for Rab21, although in the case of Rab21 it appears that it functions in a ligand-independent manner as well, enhancing the degradation of unliganded EGFR (Yang et al., 2012).

The exact mechanism by which Rab31 is engaged on early endosomes remains to be fully elucidated. GAPex5 may be important for this process. GAPex5 has been shown to bind to EGFR via Cbl (Su et al., 2007), an E3 ubiqutin ligase responsible for the ubiquitination of EGFR, which serves as both an internalisation and degradation signal. It is possible that the presence of GAPex5 on EGFR is responsible for recruiting and activating Rab31 from the cytosol onto EGFR-carrying endosomes. Once there, activated Rab31 can engage its effectors such as EEA1. We also show in Section 4.2 that Rab31 plays a role downstream of Rab5 in the endocytic trafficking of EGFR, and we speculate that Rab31 might perhaps be recruited to the EGFR trafficking complex subsequent to the involvement of Rab5. As EEA1 is also a Rab5 effector, Rab5 may be responsible for first engaging EEA1 onto EGFR-carrying endosomes, which then later interacts with the activated Rab31. How the Rab31/EEA1/EGFR trafficking complex subsequently mediates the movement of ligand-bound EGFR from early to late endosomes also remains to be explored. EEA1 may perhaps be subsequently involved in the recruitment of other late endosome markers, or in facilitating the tethering of endosomes. Fig. 7.1 illustrates the possible interactions which mediate the role of Rab31 in ligand-bound EGFR trafficking.

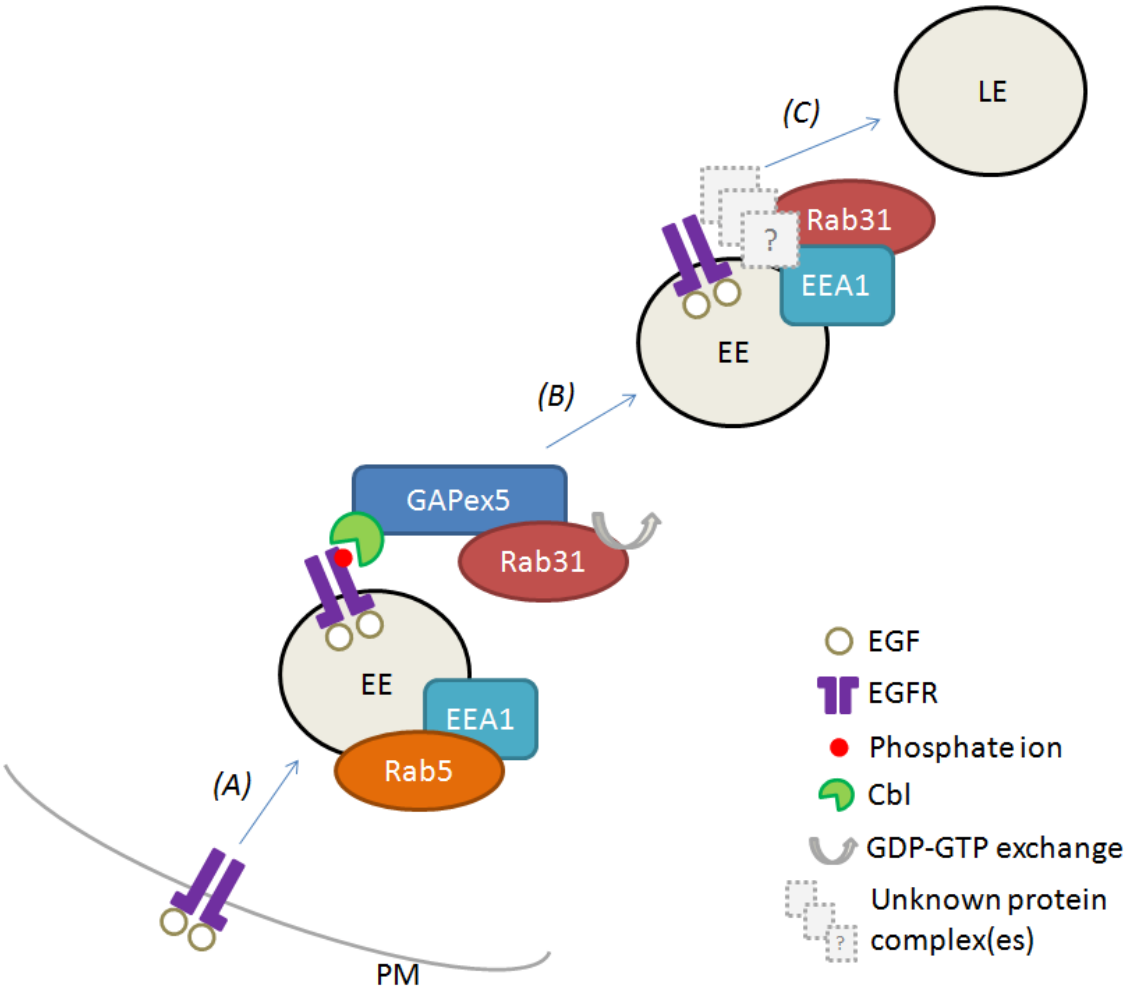


Fig. 7.1. Illustration of Rab31 interactions in ligand-bound EGFR trafficking
A) Ligand-bound EGFR is internalised into early endosomes (EE) from the plasma membrane (PM). EEA1 and Rab5 may already be present on the EE.
B) GAPex5 is bound to EGFR via Cbl. GAPex5 stimulates Rab31 GDP-GTP exchange, allowing Rab31 to engage its effector, EEA1 on the endosome.
C) Subsequently, other complexes, together with Rab31-EEA1-EGFR, may mediate the movement to late endosomes.
For simplicity only relevant protein complexes on the ligand-bound EGFR is shown, and only one phosphorylation site is shown.

We also show in Section 4.3 that Rab31 may indirectly impinge on the recycling of ligand-bound EGFR. Since Rab31 appears to mediate the trafficking of ligand-bound EGFR between early and late endosomes, Rab31 depletion would result in a decrease in channelling to the late endosome-lysosome pathway and instead shunts more ligand-bound EGFR to the recycling endosomes, and the converse could be expected for Rab31 overexpression. The extent to which Rab31 determines the rates of recycling versus degradation remains to be fully explored. Several other mechanisms have been identified which regulate the levels of EGFR recycling and degradation that occurs in the cell. For example, it was shown that ligand-bound EGFR can be internalised by both clathrin-mediated endocytosis (CME) and clathrin-independent mechanisms, with CME predominating the response to low levels of EGF, which in turn was linked to higher levels (~70%) of recycling compared to degradation (Sigismund et al., 2008). It is not yet known whether Rab31 influences the channelling of ligand-bound EGFR in the endosomes downstream of either or both CME and non CME-mediated pathways.

We provide evidence in Section 5.3 that the role of Rab31 in EGFR trafficking is mediated by the GEF GAPex5, but not RIN3. Instead, the loss of RIN3 appears to affect the localisation of M6PR, in cells with Rab31 overexpression. The exact reasons for this remain to be explored, but may be linked to observations by other groups that Rab31 also participates in the anterograde transport of M6PR from the TGN (Rodriguez-Gabin et al., 2001; Rodriguez-Gabin et al., 2009). Regardless, our observation highlights the importance of GEFs in mediating the specificity of Rab localisation and function. This is particularly significant given that we observed RIN3 to be faithfully localised to the TGN, but GAPex5 to have a more cytosolic localisation (Section 3.2). An example of different GEFs serving to activate different

roles of Rabs can be found in the DENN-domain containing connecdenns, which serve as GEFs for Rab35 (Marat and McPherson, 2010). Rab35 has a range of activities on the plasma membrane, clathrin coated pits, and endosomes, and regulates endocytic recycling and actin remodelling. Connecdenn 1 and 2 are localised to punctate structures and are believed to activate Rab35's role on endosomes, while connecdenn 3 has a unique actin-binding domain not found in the other two, which enables it to mediate Rab35's actin-related activity. Connecdenn 3 is first recruited to actin, after which it mediates GDP-to-GTP exchange on Rab35, enabling Rab35 to recruit its effector, fascin, resulting in the bundling of actin filaments (Marat et al., 2012). As another example, Rab5's GDP-GTP exchange can be aided by Rabex5 on endosomes, which is stabilized by its ability to interact with Rabaptin5, whereas RIN1 has been accorded a more specific role in regulating Rab5-mediated endocytosis of EGFR, due to the presence of SH2 and Ras association (RA) domains (van der Bliek, 2005; Carney et al., 2006). From these examples, it is evident that further exploration into the different regulatory domains on GAPex5 and RIN3, as well as other GEFs of Rab31 that come to light, will be instrumental in delineating the different roles of Rab31 in regulating TGN- and endosomal- related trafficking.

In Chapter 6 we explored the physiological relevance of Rab31 by showing that Rab31 plays a role in the differentiation of neural progenitor cells (NPCs). Rab31 is expressed in NPCs in the mouse brain. In culture, Rab31 is found in undifferentiated neural progenitor cells, and manipulation of Rab31 levels affects the subsequent differentiation of these cells into those of the astroglial lineage. This is the first time Rab31 has been shown to play a role in neural progenitor cells, and is, to our knowledge, the first indication for a role of a Rab GTPase in astroglia differentiation. Whether the role of Rab31 in NPCs is linked to its role in the trafficking of EGFR

remains to be fully explored. Manipulation of the known GEFs of Rab31 and tracing the movement of EGFR after an EGF pulse in NPCs might give some insight into how Rab31 activity is important to these cells.

Additionally, the reason for the high levels of Rab31 seen in a subset of astrocytes in our adult mouse brain cryosection and differentiated NPC culture remains to be explored. While matured astrocytes are not normally EGF-responsive, trafficking of other receptors or cell surface proteins do take place, in which Rab31 could play an important role. For example, astrocytes carry the p75 neurotrophin receptor (p75 ^{NTR}) which is trafficked in response to ligand binding (Cragnolini et al., 2009). p75 ^{NTR} was recently shown to interact with Rab31, in the context of adipocytes (Baeza-Raja et al., 2012). Although this occurred in a neurotrophin-independent manner and does not involve the trafficking of the receptor, it is conceivable that in the context of astrocytes, Rab31 might be responsible in regulating the trafficking of p75 ^{NTR}. Increasing evidence suggests that Rabs mediate more than one role in the cell, such as the example of Rab35 discussed above. As such, it is not surprising that Rab31 would also be associated with different roles in different cell types.

In summary, we have observed the two potential roles of Rab31 in EGFR trafficking and neural progenitor cell differentiation. These are likely to impact the study of both neurogenesis and tumourigenesis, as discussed in the following section.

7.2 Applications and implications of our findings

Neurogenesis serves learning and memory, and also has roles for repair in pathological states (Duan et al., 2008), and as such has many important applications. For example, it has been argued and hoped that NPCs have a large potential in CNS

transplantation therapy. However, one problem frequently encountered is that astrogliosis is often triggered in an injured CNS environment (Chen and Swanson, 2003; Floyd, 2012). Astrogliosis results in changes to the molecular expression and morphology of astrocytes, which result in hyper-proliferation and eventual scar formation. This in turn inhibits axon or neuronal regeneration. Our results indicate that depletion of Rab31 in NPCs can attenuate astroglia formation. It is therefore conceivable that manipulation of Rab31 levels in NPCs before transplantation into a site of traumatic CNS injury could aid in improving neuronal differentiation and neurite growth. Moreover, it has been shown that blocking EGFR activation in astrocytes is beneficial for neuronal survival in cases of traumatic CNS injury, as it minimises the activation of reactive astrocytes (Liu and Neufeld, 2007). Since Rab31 has been associated with EGFR trafficking, manipulation of Rab31 levels in mature astrocytes might also prove beneficial.

It has also been shown that myelin-associated inhibitors and chondroitin sulphate proteoglycans from the glial scar, which act to inhibit axonal regeneration, act on neurons through EGFR signalling pathways (Koprivica, 2005). Although our current research has focussed on Rab31 in NPCs and astrocytes rather than neurons, and the extent of Rab31 expression in neurons is unknown, it is also possible that manipulation of EGFR trafficking pathways via Rab31 in neurons might be one way to overcome the inhibition posed by myelin and glial scar inhibitors.

The flipside to neurogenesis is tumourigenesis, which also has strong links to EGFR signalling. Glioblastoma multiforme (GBM) is a form of brain cancer caused by malignant glioma. NPCs are believed to be the cell of origin of GBM tumour stem cells. EGFR has been frequently found to be amplified in 45% of GBM. Mutations have also been associated with GBM, the most common being EGFRvIII, a truncation

in the extracellular domain, resulting in a ligand-independent, constitutively activated kinase (Ayuso-Sacido et al., 2006). As such, many cancer therapies rely on the blocking of EGFR signalling. Antibodies such as Cetuximab have been developed, as well as small molecule inhibitors of EGFR tyrosine kinase, such as Gefitinib, which binds to the ATP-binding site of the kinase. In studies done in U87 cells, a glioblastoma cell line, the authors identified Mig6 as a potential tumour suppressor. Mig6 enhances EGFR trafficking to the late endosome and lysosome by binding to Syntaxin 8, a SNARE protein that mediates late endosome trafficking (Ying et al., 2010). As Rab31 acts on a similar step in EGFR trafficking, it is conceivable that it may also be important in tumourigenesis, perhaps as a tumour suppressor.

Interestingly, however, Rab31 has been shown to be of value as a biomarker in GBM (Serão et al., 2011), and was recently found to be upregulated in GBM (Kunkle et al., 2013). Rab31 was also shown to have prognostic values in breast cancer and elevated levels have been associated with poorer survival outcomes (Kotzsch et al., 2008; Kotzsch et al., 2011). Rab31 is also differentially overexpressed in estrogen receptor α (ERα)-positive breast carcinomas (Abba et al., 2005). As such, Rab31 appears, in fact, to be elevated in many cancers. Several reasons for this have recently come to light. Firstly, the Rab31 promoter has been shown to have an estrogen receptor responsive element, and may therefore be controlled by associated trans factors. Indeed, it has been shown that mucin-1 (MUC1), which is overexpressed in many human cancer cells, forms a complex with ERα that both stabilises ERα by blocking its ubiquitination and activates Rab31 transcription (Jin et al., 2012), thus suggesting that Rab31 may be an oncogenic driver for ERα breast cancers. Interestingly, the study also found that Rab31, in turn, resulted in elevated levels of MUC1, itself a multifunctional oncoprotein (Kufe, 2013), by decreasing the lysosomal

degradation of MUC1 via an as-yet-undefined mechanism, thus forming a potential auto-inductive loop. In the future we would seek to investigate how Rab31 can influence the trafficking of MUC1.

On the other hand, other studies have shown that overexpression of Rab31 in breast cancer cell lines switched these from an invasive to a proliferative phenotype, and significantly reduced lung cancer metastasis in a mouse xenograft model (Grismayer et al., 2012). This again highlights the complexity of responses in manipulation of a single protein involved in the multifaceted pathways of oncogenesis.

How might we reconcile the evidence that Rab31 may be an oncogenesis-related protein with our own observations that in A431 and HeLa cells, Rab31 appears to downregulate EGFR (an important oncogenesis-related signal), via its role in the early-to-late endosome trafficking of ligand-bound EGFR? One reason could be due to differences in cell type, and the associated differences in levels of Rab31 GEFs like GAPex5, and also hitherto unknown GAPs. Rab25, for example, has also been alternately identified as an oncogene or a tumour suppressor, and the general consensus is that its role in cancer is largely context dependent, possibly in part due to the expression levels of its effector, Rab Coupling Protein (RCP) (Tang, 2010; Mitra et al., 2012). Likewise, in contrast to breast cancer, Rab31 was shown to have no prognostic value in ovarian cancer (Kotzsch et al., 2008; Kotzsch et al., 2011). Also, multiple mutations in cancer cell lines, especially in EGFR itself, may alter the way in which Rab31 affects EGFR trafficking and signalling. Moreover, as described above, Rab31 also affects MUC1 levels (Jin et al., 2012). As MUC1 itself has also been shown to bind EGFR and potentiate its signalling by reducing ubiquitination (Pochampalli et al., 2007), the net effect of Rab31, MUC1 and EGFR in pre-cancerous and cancerous cells is currently unclear. Fig. 7.2. highlights some of the known interactions between

Rab31, MUC1 and EGFR. Manipulation of Rab31 may, in the future, be a tenable therapeutic strategy against a variety of cancers.

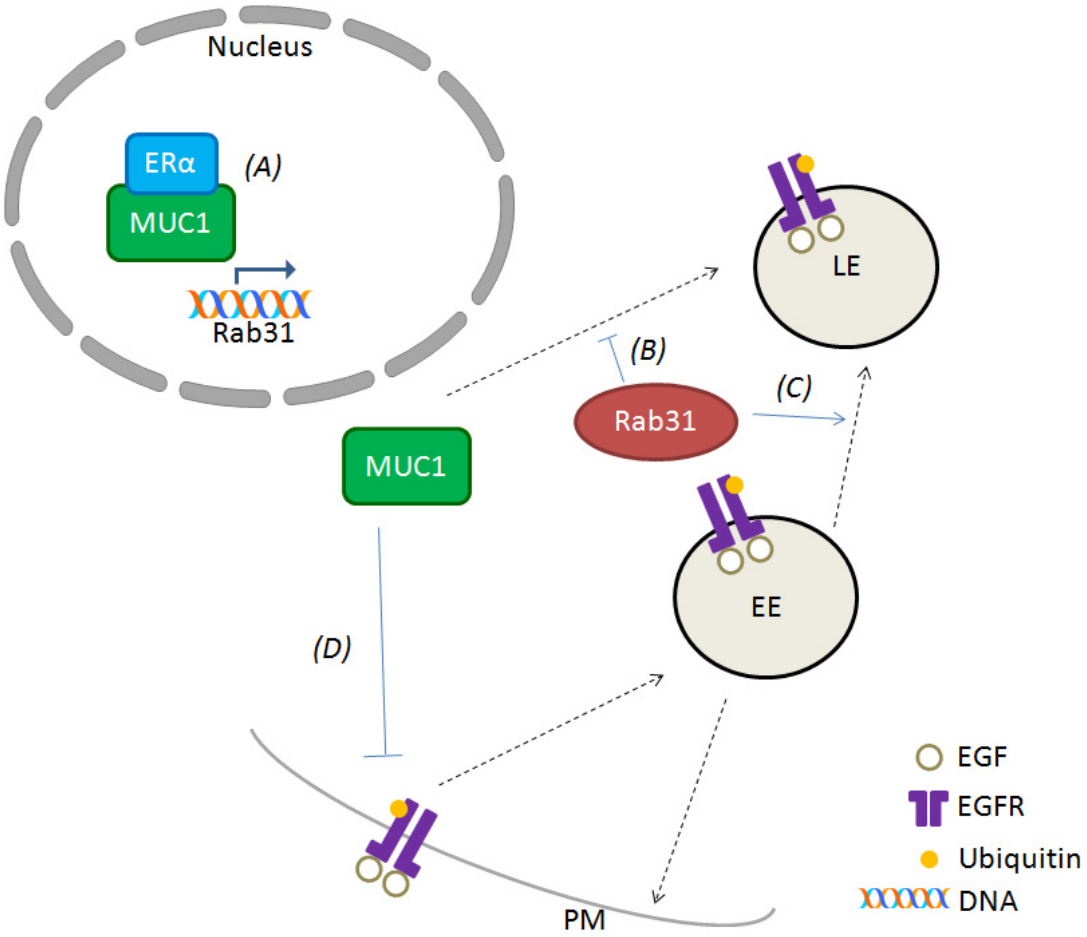


Fig. 7.2. Interactions between Rab31, EGFR and MUC1
(A) In the nucleus, MUC1 enhances the transcription of Rab31 by stabilising the ERα transcription factor.
(B) In the cytosol, Rab31 enhances the stability of MUC1 by decreasing its lysosomal degradation.
(C) Rab31 facilitates the trafficking of ligand-bound EGFR from early to late endosome.
(D) MUC1 potentiates ligand-bound EGFR signalling by reducing the ubiquitination and hence degradation of EGFR, thus enhancing the recycling of EGFR to the cell surface.
PM: Plasma membrane, EE: early endosome, LE: late endosome.
For simplicity only one ubiquitination site is shown.

Because of the multivariate effects of EGFR signalling in neurodevelopment as well as tumour progression, manipulation of EGFR signalling in therapeutic strategies must be tempered with caution and a better understanding of how the signalling is regulated. To this end, deciphering the exact cellular roles and activities of Rab31 is therefore important.

In conclusion, our results indicate that Rab31 is a hitherto underappreciated and important player in the endocytic trafficking of EGFR which functions in an EGFR trafficking complex together EEA1 and GAPex5. It could conceivably be anticipated that alterations in Rab31 expression or activity, which has been documented in various cancers (Kotzsch et al., 2008; Grismayer et al., 2012; Kunkle et al., 2013), will affect EGFR trafficking and signalling in ways that may contribute to malignancy and metastasis. Rab31 also appears to have a role in astroglia differentiation, perhaps through regulation of EGFR trafficking and signaling, a notion that has yet to be fully explored. Further work along these lines would prove to be fertile pursuits.

References

Abba MC, Hu Y, Sun H, Drake JA, Gaddis S, Baggerly K, Sahin A, Aldaz CM. 2005. Gene expression signature of estrogen receptor α status in breast cancer. BMC Genomics 6:37-37.

Aivazian D, Serrano RL, Pfeffer S. 2006. TIP47 is a key effector for Rab9 localization. J Cell Biol 173:917-926.

Alagappan D, Lazzarino DA, Felling RJ, Balan M, Kotenko SV, Levison SW. 2009. Brain injury expands the numbers of neural stem cells and progenitors in the SVZ by enhancing their responsiveness to EGF. ASN neuro 1

Ali BR, Wasmeier C, Lamoreux L, Strom M, Seabra MC. 2004. Multiple regions contribute to membrane targeting of Rab GTPases. J Cell Sci 117:6401-6412.

Allaire PD, Marat AL, Dall'Armi C, Di Paolo G, McPherson PS, Ritter B. 2010. The Connecdenn DENN domain: a GEF for Rab35 mediating cargo-specific exit from early endosomes. Mol Cell 37:370-382.

Allan BB. 2000. Rab1 Recruitment of p115 into a cis-SNARE Complex: Programming Budding COPII Vesicles for Fusion. Science 289:444-448.

Alvarez-Buylla A, García-Verdugo JM, Tramontin AD. 2001. A unified hypothesis on the lineage of neural stem cells. Nat Rev Neurosci 2:287-293.

Ayuso-Sacido A, Graham C, Greenfield JP, Boockvar JA. 2006. The duality of epidermal growth factor receptor (EGFR) signaling and neural stem cell phenotype: cell enhancer or cell transformer? Curr Stem Cell Res Ther 1:387-394.

Ayuso-Sacido A, Moliterno JA, Kratovac S, Kapoor GS, O'Rourke DM, Holland EC, García-Verdugo JM, Roy NS, Boockvar JA. 2010. Activated EGFR signaling increases proliferation, survival, and migration and blocks neuronal differentiation in post-natal neural stem cells. J Neurooncol 97:323-337.

Baeza-Raja B, Li P, Le Moan N, Sachs BD, Schachtrup C, Davalos D, Vagena E, Bridges D, Kim C, Saltiel AR, Olefsky JM, Akassoglou K. 2012. p75 neurotrophin receptor regulates glucose homeostasis and insulin sensitivity. Proceedings of the National Academy of Sciences 109:5838-5843.

Barbero P, Bittova L, Pfeffer SR. 2002. Visualization of Rab9-mediated vesicle transport from endosomes to the trans-Golgi in living cells. J Cell Biol 156:511-518.

Barbieri MA, Roberts RL, Gumusboga A, Highfield H, Alvarez-Dominguez C, Wells A, Stahl PD. 2000. Epidermal growth factor and membrane trafficking. EGF receptor activation of endocytosis requires Rab5a. J Cell Biol 151:539-550.

Barral DC, Ramalho J. 2002. Functional redundancy of Rab27 proteins and the pathogenesis of Griscelli syndrome. Journal of Clinical Investigation 110:247-257.

Beard M, Satoh A, Shorter J, Warren G. 2005. A cryptic Rab1-binding site in the p115 tethering protein. J Biol Chem 280:25840-25848.

Bem D, Yoshimura SI, Nunes-Bastos R, Bond FF, Kurian MA, Rahman F, Handley MT, Hadzhiev Y, Masood I, Straatman-Iwanowska AA. 2011. Loss-of-function mutations in RAB18 cause Warburg micro syndrome. Am J Hum Genet 88:499-507.

Ben-Salem S, Begum M, Ali B, Al-Gazali L. 2013. A Novel Aberrant Splice Site Mutation in RAB23 Leads to an Eight Nucleotide Deletion in the mRNA and Is Responsible for Carpenter Syndrome in a Consanguineous Emirati Family. Molecular syndromology 3:255-261.

Bliss JM, Venkatesh B, Colicelli J. 2006. The RIN family of Ras effectors. Methods Enzymol 407:335-344.

Blumer J, Rey J, Dehmelt L, Mazel T, Wu YW, Bastiaens P, Goody RS, Itzen A. 2013. RabGEFs are a major determinant for specific Rab membrane targeting. (2). J Cell Biol 200:287-300.

Bravo-Cordero JJ, Marrero-Diaz R, Megías D, Genís L, García-Grande A, García MA, Arroyo AG, Montoya MC. 2007. MT1-MMP proinvasive activity is regulated by a novel Rab8-dependent exocytic pathway. EMBO J 26:1499-1510.

Brown TC, Tran IC, Backos DS, Esteban JA. 2005. NMDA receptor-dependent activation of the small GTPase Rab5 drives the removal of synaptic AMPA receptors during hippocampal LTD. Neuron 45:81-94.

Bucci C, Parton RG, Mather IH, Stunnenberg H, Simons K, Hoflack B, Zerial M. 1992. The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway. Cell 70:715-728.

Burrows RC, Wancio D, Levitt P, Lillien L. 1997. Response diversity and the timing of progenitor cell maturation are regulated by developmental changes in EGFR expression in the cortex. Neuron 19:251-267.

Calero M, Chen CZ, Zhu W, Winand N, Havas KA, Gilbert PM, Burd CG, Collins RN. 2003. Dual prenylation is required for Rab protein localization and function. Mol Biol Cell 14:1852-1867.

Carney DS, Davies BA, Horazdovsky BF. 2006. Vps9 domain-containing proteins: activators of Rab5 GTPases from yeast to neurons. Trends Cell Biol 16:27-35.

Caswell PT, Spence HJ, Parsons M, White DP, Clark K, Cheng KW, Mills GB, Humphries MJ, Messent AJ, Anderson KI, McCaffrey MW, Ozanne BW, Norman JC. 2007. Rab25 associates with alpha5beta1 integrin to promote invasive migration in 3D microenvironments. Dev Cell 13:496-510.

Ceresa BP. 2006. Regulation of EGFR endocytic trafficking by rab proteins. Histol Histopathol 21:987-993.

Ceresa BP, Bahr SJ. 2006. rab7 activity affects epidermal growth factor:epidermal growth factor receptor degradation by regulating endocytic trafficking from the late endosome. J Biol Chem 281:1099-1106.

Chavrier P, Gorvel JP, Stelzer E, Simons K, Gruenberg J, Zerial M. 1991. Hypervariable C-terminal domain of rab proteins acts as a targeting signal. Nature 353:769-772.

Chen Y, Swanson RA. 2003. Astrocytes and brain injury. Journal of Cerebral Blood Flow \& Metabolism 23:137-149.

Chen D, Guo J, Miki T, Tachibana M, Gahl WA. 1996. Molecular cloning of two novel rab genes from human melanocytes. Gene 174:129-134.

Chen PI, Kong C, Su X, Stahl PD. 2009. Rab5 isoforms differentially regulate the trafficking and degradation of epidermal growth factor receptors. J Biol Chem 284:30328-30338.

Chen X, Wang Z. 2001. Regulation of intracellular trafficking of the EGF receptor by Rab5 in the absence of phosphatidylinositol 3-kinase activity. EMBO Rep 2:68-74.

Cheng KW, Lahad JP, Kuo WL, Lapuk A, Yamada K, Auersperg N, Liu J, Smith-McCune K, Lu KH, Fishman D, Gray JW, Mills GB. 2004. The RAB25 small GTPase determines aggressiveness of ovarian and breast cancers. Nat Med 10:1251-1256.

Chia WJ, Tang BL. 2009. Emerging roles for Rab family GTPases in human cancer. Biochim Biophys Acta 1795:110-116.

Chiariello M, Bruni CB, Bucci C. 1999. The small GTPases Rab5a, Rab5b and Rab5c are differentially phosphorylated in vitro. FEBS Lett 453:20-24.

Cho JM, Shin YJ, Park JM, Kim J, Lee MY. 2013. Characterization of nestin expression in astrocytes in the rat hippocampal CA1 region following transient forebrain ischemia. Anatomy & cell biology 46:131-140.

Chojnacki A, Weiss S. 2008. Production of neurons, astrocytes and oligodendrocytes from mammalian CNS stem cells. Nat Protoc 3:935-940.

Christoforidis S, McBride HM, Burgoyne RD, Zerial M. 1999. The Rab5 effector EEA1 is a core component of endosome docking. Curr Opin Cell Biol 397:621-625.

Chua CEL, Tang BL. 2011. Rabs, SNAREs and α-synuclein - Membrane trafficking defects in synucleinopathies. Brain Res Rev 67:268-281.

Cogli L, Progida C, Thomas CL, Spencer-Dene B, Donno C, Schiavo G, Bucci C. 2013. Charcot-Marie-Tooth type 2B disease-causing RAB7A mutant proteins show altered interaction with the neuronal intermediate filament peripherin. Acta Neuropathol 125:257-272.

Colicelli J. 2004. Human RAS Superfamily Proteins and Related GTPases. Science's STKE 2004:re13-re13.

Cragnolini AB, Huang Y, Gokina P, Friedman WJ. 2009. Nerve growth factor attenuates proliferation of astrocytes via the p75 neurotrophin receptor. Glia 57:1386-1392.

Cullis DN, Philip B, Baleja JD, Feig LA. 2002. Rab11-FIP2, an adaptor protein connecting cellular components involved in internalization and recycling of epidermal growth factor receptors. J Biol Chem 277:49158-49166.

Damen E, Krieger E, Nielsen JE, Eygensteyn J, Van Leeuwen JEM. 2006. The human Vps29 retromer component is a metallo-phosphoesterase for a cation-independent mannose 6-phosphate receptor substrate peptide. Biochem J 398:399-409.

de Hoop MJ, Huber LA, Stenmark H, Williamson E, Zerial M, Parton RG, Dotti CG. 1994. The involvement of the small GTP-binding protein Rab5a in neuronal endocytosis. Neuron 13:11-22.

De Renzis S, Sönnichsen B, Zerial M. 2002. Divalent Rab effectors regulate the sub-compartmental organization and sorting of early endosomes. Nat Cell Biol 4:124-133.

Delprato A, Lambright DG. 2007. Structural basis for Rab GTPase activation by VPS9 domain exchange factors. Nature Structural & Molecular Biology 14:406-412.

Delprato A, Merithew E, Lambright DG. 2004. Structure, Exchange Determinants, and Family-Wide Rab Specificity of the Tandem Helical Bundle and Vps9 Domains of Rabex-5. Cell 118:607-617.

Deneka M, Neeft M, van der Sluijs P. 2003. Regulation of membrane transport by rab GTPases. Crit Rev Biochem Mol Biol 38:121-142.

Di Fiore PP, De Camilli P. 2001. Endocytosis and signaling. an inseparable partnership. Cell 106:1-4.

Dinneen JL, Ceresa BP. 2004. Expression of dominant negative rab5 in HeLa cells regulates endocytic trafficking distal from the plasma membrane. Exp Cell Res 294:509-522.

Dirac-Svejstrup AB, Sumizawa T, Pfeffer SR. 1997. Identification of a GDI displacement factor that releases endosomal Rab GTPases from Rab-GDI. EMBO J 16:465-472.

Doetsch F. 2003. The glial identity of neural stem cells. Nat Neurosci 6:1127-1134.

Duan X, Kang E, Liu CY, Ming GL, Song H. 2008. Development of neural stem cell in the adult brain. Curr Opin Neurobiol 18:108-115.

Dumas JJ, Merithew E, Sudharshan E, Rajamani D, Hayes S, Lawe D, Corvera S, Lambright DG. 2001. Multivalent endosome targeting by homodimeric EEA1. Mol Cell 8:947-958.

Echard A. 1998. Interaction of a Golgi-Associated Kinesin-Like Protein with Rab6. Science 279:580-585.

Egami Y, Araki N. 2008. Characterization of Rab21-positive tubular endosomes induced by PI3K inhibitors. Exp Cell Res 314:729-737.

Feng Y, Press B, Wandinger-Ness A. 1995. Rab 7: an important regulator of late endocytic membrane traffic. J Cell Biol 131:1435-1452.

Ferrándiz-Huertas C, Fernández-Carvajal A, Ferrer-Montiel A. 2011. Rab4 interacts with the human P-glycoprotein and modulates its surface expression in multidrug resistant K562 cells. International Journal of Cancer 128:192-205.

Filippov V, Kronenberg G, Pivneva T, Reuter K, Steiner B, Wang LP, Yamaguchi M, Kettenmann H, Kempermann G. 2003. Subpopulation of nestin-expressing progenitor cells in the adult murine hippocampus shows electrophysiological and morphological characteristics of astrocytes. Molecular and Cellular Neuroscience 23:373-382.

Floyd CL. 2012. Assessments of Reactive Astrogliosis Following CNS Injuries. Animal Models of Acute Neurological Injuries II :53-69.

Fukuda M. 2011. TBC proteins: GAPs for mammalian small GTPase Rab? Biosci Rep 31:159-168.

Fukui K, Tamura S, Wada A, Kamada Y, Igura T, Kiso S, Hayashi N. 2007. Expression of Rab5a in hepatocellular carcinoma: Possible involvement in epidermal growth factor signaling. Hepatology research : the official journal of the Japan Society of Hepatology 37:957-965.

Gabernet-Castello C, O'Reilly AJ, Dacks JB, Field MC. 2013. Evolution of Tre-2/Bub2/Cdc16 (TBC) Rab GTPase-activating proteins. Mol Biol Cell 24:1574-1583.

García-Verdugo JM, Doetsch F, Wichterle H, Lim DA, Alvarez-Buylla A. 1998. Architecture and cell types of the adult subventricular zone: in search of the stem cells. J Neurobiol 36:234-248.

Gerondopoulos A, Langemeyer L, Liang JR, Linford A, Barr FA. 2012. BLOC-3 mutated in Hermansky-Pudlak syndrome is a Rab32/38 guanine nucleotide exchange factor. Curr Biol 22:2135-2139.

Giannandrea M, Bianchi V, Mignogna ML, Sirri A, Carrabino S, D'Elia E, Vecellio M, Russo S, Cogliati F, Larizza L. 2010. Mutations in the small GTPase gene RAB39B are responsible for X-linked mental retardation associated with autism, epilepsy, and macrocephaly. Am J Hum Genet 86:185-195.

Gomes AQ, Ali BR, Ramalho JS, Godfrey RF, Barral DC, Hume AN, Seabra MC. 2003. Membrane targeting of Rab GTPases is influenced by the prenylation motif. Mol Biol Cell 14:1882-1899.

Gómez-Pinilla F, Knauer DJ, Nieto-Sampedro M. 1988. Epidermal growth factor receptor immunoreactivity in rat brain. Development and cellular localization. Brain Res 438:385-390.

Goody RS, Rak A, Alexandrov K. 2005. The structural and mechanistic basis for recycling of Rab proteins between membrane compartments. Cell Mol Life Sci 62:1657-1670.

Grabski R, Balklava Z, Wyrozumska P, Szul T, Brandon E, Alvarez C, Holloway ZG, Sztul E. 2012. Identification of a functional domain within the p115 tethering factor that is required for Golgi ribbon assembly and membrane trafficking. J Cell Sci 125:1896-1909.

Gregg C, Weiss S. 2003. Generation of functional radial glial cells by embryonic and adult forebrain neural stem cells. J Neurosci 23:11587-11601.

Grismayer B, Sölch S, Seubert B, Kirchner T, Schäfer S, Baretton G, Schmitt M, Luther T, Krüger A, Kotzsch M, Magdolen V. 2012. Rab31 expression levels modulate tumor-relevant characteristics of breast cancer cells. 11:62.

Grosshans BL, Ortiz D, Novick P. 2006. Rabs and their effectors: achieving specificity in membrane traffic. Proc Natl Acad Sci U S A 103:11821-11827.

Gu F, Crump C, Thomas G. 2001. Trans-Golgi network sorting. Cell Mol Life Sci 58:1067-1084.

Herrmann H, Aebi U. 2000. Intermediate filaments and their associates: multi-talented structural elements specifying cytoarchitecture and cytodynamics. Curr Opin Cell Biol 12:79-90.

Horiuchi H, Lippé R, McBride HM, Rubino M, Woodman P, Stenmark H, Rybin V, Wilm M, Ashman K, Mann M, Zerial M. 1997. A novel Rab5 GDP/GTP exchange factor complexed to Rabaptin-5 links nucleotide exchange to effector recruitment and function. Cell 90:1149-1159.

Huang F, Khvorova A, Marshall W, Sorkin A. 2004. Analysis of clathrin-mediated endocytosis of epidermal growth factor receptor by RNA interference. J Biol Chem 279:16657-16661.

Hunker C, Galvis A, Kruk I, Giambini H, Veisaga M, Barbieri M. 2006. Rab5-activating protein 6, a novel endosomal protein with a role in endocytosis. Biochem Biophys Res Commun 340:967-975.

Hutagalung AH, Novick PJ. 2011. Role of Rab GTPases in membrane traffic and cell physiology. Physiol Rev 91:119-149.

Ihrie RA, Alvarez-Buylla A. 2008. Cells in the astroglial lineage are neural stem cells. Cell Tissue Res 331:179-191.

Imura T, Kornblum HI, Sofroniew MV. 2003. The predominant neural stem cell isolated from postnatal and adult forebrain but not early embryonic forebrain expresses GFAP. J Neurosci 23:2824-2832.

Jackson EL, Garcia-Verdugo JM, Gil-Perotin S, Roy M, Quinones-Hinojosa A, VandenBerg S, Alvarez-Buylla A. 2006. PDGFR alpha-positive B cells are neural stem cells in the adult SVZ that form glioma-like growths in response to increased PDGF signaling. Neuron 51:187-199.

Jenkins D, Seelow D, Jehee FS, Perlyn CA, Alonso LG, Bueno DF, Donnai D, Josifiova D, Mathijssen IM, Morton JE, Helene Ørstavik K, Sweeney E, Wall SA, Marsh JL, Nürnberg P, Rita Passos-Bueno M, Wilkie AO. 2007. RAB23 mutations in Carpenter syndrome imply an unexpected role for hedgehog signaling in cranial-suture development and obesity. Am J Hum Genet 80:1162-1170.

Jin C, Rajabi H, Pitroda S, Li A, Kharbanda A, Weichselbaum R, Kufe D, Katz E. 2012. Cooperative interaction between the MUC1-C oncoprotein and the Rab31 GTPase in estrogen receptor-positive breast cancer cells. PloS one 7:e39432.

Jordens I, Fernandez-Borja M, Marsman M, Dusseljee S, Janssen L, Calafat J, Janssen H, Wubbolts R, Neefjes J. 2001. The Rab7 effector protein RILP controls lysosomal transport by inducing the recruitment of dynein-dynactin motors. Curr Biol 11:1680-1685.

Jozic I, Saliba SC, Alejandro Barbieri M. 2012. Effect of EGF-receptor tyrosine kinase inhibitor on Rab5 function during endocytosis. Arch Biochem Biophys 525:16-24.

Kajiho H, Sakurai K, Minoda T, Yoshikawa M, Nakagawa S, Fukushima S, Kontani K, Katada T. 2011. Characterization of RIN3 as a guanine nucleotide exchange factor for the Rab5 subfamily GTPase Rab31. J Biol Chem 286:24364-24373.

Kanno E, Ishibashi K, Kobayashi H, Matsui T, Ohbayashi N, Fukuda M. 2010. Comprehensive Screening for Novel Rab-Binding Proteins by GST Pull-Down Assay Using 60 Different Mammalian Rabs‡. Traffic 11:491-507.

Kauppi M, Simonsen A, Bremnes B. 2002. The small GTPase Rab22 interacts with EEA1 and controls endosomal membrane trafficking. J Cell Sci 115:899-911.

King GJ, Stockli J, Hu SH, Winnen B, Duprez WGA, Meoli CC, Junutula JR, Jarrott RJ, James DE, Whitten AE, Martin JL. 2012. Membrane curvature protein exhibits interdomain flexibility and binds a small GTPase. J Biol Chem 287:40996-41006.

Klöpper TH, Kienle N, Fasshauer D, Munro S. 2012. Untangling the evolution of Rab G proteins: implications of a comprehensive genomic analysis. BMC Biol 10:71.

Koprivica V. 2005. EGFR activation mediates inhibition of axon regeneration by myelin and chondroitin sulfate proteoglycans. Science 310:106-110.

Kornblum HI, Hussain RJ, Bronstein JM, Gall CM, Lee DC, Seroogy KB. 1997. Prenatal ontogeny of the epidermal growth factor receptor and its ligand, transforming growth factor alpha, in the rat brain. J Comp Neurol 380:243-261.

Kotzsch M, Dorn J, Doetzer K, Schmalfeldt B, Krol J, Baretton G, Kiechle M, Schmitt M, Magdolen V. 2011. mRNA expression levels of the biological factors uPAR, uPAR-del4/5, and Rab31, displaying prognostic value in breast cancer, are not clinically relevant in advanced ovarian cancer. Biol Chem 392:1047-1051.

Kotzsch M, Sieuwerts AM, Grosser M, Meye A, Fuessel S, Meijer-van Gelder ME, Smid M, Schmitt M, Baretton G, Luther T, Magdolen V, Foekens JA. 2008. Urokinase receptor splice variant uPAR-del4/5-associated gene expression in breast cancer: identification of rab31 as an independent prognostic factor. Breast Cancer Res Treat 111:229-240.

Kreft M, Potokar M, Stenovec M, Pangršič T, Zorec R. 2009. Regulated Exocytosis and Vesicle Trafficking in Astrocytes. Ann N Y Acad Sci 1152:30-42.

Kriegstein A, Alvarez-Buylla A. 2009. The glial nature of embryonic and adult neural stem cells. Annu Rev Neurosci 32:149-184.

Kufe DW. 2013. MUC1-C oncoprotein as a target in breast cancer: activation of signaling pathways and therapeutic approaches. Oncogene 32:1073-1081.

Kunkle BW, Yoo C, Roy D, Singh K. 2013. Reverse engineering of modified genes by bayesian network analysis defines molecular determinants critical to the development of glioblastoma. PloS one 8:e64140.

Li L. 2001. Direct interaction of Rab4 with syntaxin 4. J Biol Chem 276:5265-5273.

Lim DA, Alvarez-Buylla A. 1999. Interaction between astrocytes and adult subventricular zone precursors stimulates neurogenesis. Proc Natl Acad Sci U S A 96:7526-7531.

Lippé R, Miaczynska M, Rybin V, Runge A, Zerial M. 2001. Functional synergy between Rab5 effector Rabaptin-5 and exchange factor Rabex-5 when physically associated in a complex. Mol Biol Cell 12:2219-2228.

Liu B. 2006. Epidermal growth factor receptor activation: an upstream signal for transition of quiescent astrocytes into reactive astrocytes after neural injury. J Neurosci 26:7532-7540.

Liu B, Neufeld AH. 2004. Activation of epidermal growth factor receptors directs astrocytes to organize in a network surrounding axons in the developing rat optic nerve. Dev Biol 273:297-307.

Liu B, Neufeld AH. 2007. Activation of epidermal growth factor receptors in astrocytes: from development to neural injury. J Neurosci Res 85:3523-3529.

Liu NS, Loo LS, Loh E, Seet LF, Hong W. 2009. Participation of Tom1L1 in EGF-stimulated endocytosis of EGF receptor. EMBO J 28:3485-3499.

Liu Y, Namba T, Liu J, Suzuki R, Shioda S, Seki T. 2010. Glial fibrillary acidic protein-expressing neural progenitors give rise to immature neurons via early intermediate progenitors expressing both glial fibrillary acidic protein and neuronal markers in the adult hippocampus. Neuroscience 166:241-251.

Lodhi IJ, Chiang SH, Chang L, Vollenweider D, Watson RT, Inoue M, Pessin JE, Saltiel AR. 2007. Gapex-5, a Rab31 guanine nucleotide exchange factor that regulates Glut4 trafficking in adipocytes. Cell Metab 5:59-72.

Low WC, Yau WWY, Stanton LW, Marcy G, Goh E, Chew SY. 2012. Directing neuronal differentiation of primary neural progenitor cells by gene knockdown approach. DNA Cell Biol 31:1148-1160.

Madison DL, Krüger WH, Kim T, Pfeiffer SE. 1996. Differential expression of rab3 isoforms in oligodendrocytes and astrocytes. J Neurosci Res 45:258-268.

Mai A, Veltel S, Pellinen T, Padzik A, Coffey E, Marjomaki V, Ivaska J. 2011. Competitive binding of Rab21 and p120RasGAP to integrins regulates receptor traffic and migration. (1). J Cell Biol 194:291-306.

Marat AL, Ioannou MS, McPherson PS. 2012. Connecdenn 3/DENND1C binds actin linking Rab35 activation to the actin cytoskeleton. Mol Biol Cell 23:163-175.

Marat AL, McPherson PS. 2010. The connecdenn family, Rab35 guanine nucleotide exchange factors interfacing with the clathrin machinery. J Biol Chem 285:10627-10637.

Markgraf DF, Peplowska K, Ungermann C. 2007. Rab cascades and tethering factors in the endomembrane system. FEBS Lett 581:2125-2130.

Martinu L, Santiago-Walker A, Qi H, Chou MM. 2002. Endocytosis of epidermal growth factor receptor regulated by Grb2-mediated recruitment of the Rab5 GTPase-activating protein RN-tre. J Biol Chem 277:50996-51002.

Masui H, Castro L, Mendelsohn J. 1993. Consumption of EGF by A431 cells: evidence for receptor recycling. J Cell Biol 120:85-93.

McBride HM, Rybin V, Murphy C, Giner A, Teasdale R, Zerial M. 1999. Oligomeric complexes link Rab5 effectors with NSF and drive membrane fusion via interactions between EEA1 and syntaxin 13. (1). Cell 98:377-386.

McCaffrey MW, Bielli A, Cantalupo G, Mora S, Roberti V, Santillo M, Drummond F, Bucci C. 2001. Rab4 affects both recycling and degradative endosomal trafficking. FEBS Lett 495:21-30.

Mendoza P, Ortiz R, Diaz J, Quest AFG, Leyton L, Stupack D, Torres VA. 2013. Rab5 activation promotes focal adhesion disassembly, migration and invasiveness in tumor cells. J Cell Sci 126:3835-3847.

Merithew E, Stone C, Eathiraj S, Lambright DG. 2003. Determinants of Rab5 interaction with the N terminus of early endosome antigen 1. J Biol Chem 278:8494-8500.

Miaczynska M, Christoforidis S, Giner A, Shevchenko A, Uttenweiler-Joseph S, Habermann B, Wilm M, Parton R, Zerial M. 2004. APPL proteins link Rab5 to nuclear signal transduction via an endosomal compartment. Cell 116:445-456.

Mills IG, Urbé S, Clague MJ. 2001. Relationships between EEA1 binding partners and their role in endosome fusion. J Cell Sci 114:1959-1965.

Mishra A, Eathiraj S, Corvera S, Lambright DG. 2010. Structural basis for Rab GTPase recognition and endosome tethering by the C2H2 zinc finger of Early Endosomal Autoantigen 1 (EEA1). Proc Natl Acad Sci U S A 107:10866-10871.

Rab GTPases implicated in inherited and acquired disorders. Seminars in cell \& developmental biology; 2011. 57 p.

Mitra S, Cheng KW, Mills GB. 2012. Rab25 in Cancer: A brief update. Biochem Soc Trans 40:1404-1408.

Morozova N, Liang Y, Tokarev AA, Chen SH, Cox R, Andrejic J, Lipatova Z, Sciorra VA, Emr SD, Segev N. 2006. TRAPPII subunits are required for the specificity switch of a Ypt-Rab GEF. Nat Cell Biol 8:1263-1269.

Moyer BD, Allan BB, Balch WE. 2001. Rab1 Interaction with a GM130 Effector Complex Regulates COPII Vesicle cis-Golgi Tethering. Traffic 2:268-276.

Ng EL, Ng JJ, Liang F, Tang BL. 2009. Rab22B is expressed in the CNS astroglia lineage and plays a role in epidermal growth factor receptor trafficking in A431 cells. J Cell Physiol 221:716-728.

Ng EL, Tang BL. 2008. Rab GTPases and their roles in brain neurons and glia. Brain Res Rev 58:236-246.

Ng EL, Wang Y, Tang BL. 2007. Rab22B's role in trans-Golgi network membrane dynamics. Biochem Biophys Res Commun 361:751-757.

Nottingham RM, Pfeffer SR. 2009. Defining the boundaries: Rab GEFs and GAPs. Proc Natl Acad Sci U S A 106:14185-14186.

Ohya T, Miaczynska M, Coskun U, Lommer B, Runge A, Drechsel D, Kalaidzidis Y, Zerial M. 2009. Reconstitution of Rab- and SNARE-dependent membrane fusion by synthetic endosomes. Nature 459:1091-1097.

Oiso N, Riddle SR, Serikawa T, Kuramoto T, Spritz RA. 2004. The rat Ruby (R) locus is Rab38: identical mutations in Fawn-hooded and Tester-Moriyama rats derived from an ancestral Long Evans rat sub-strain. Mamm Genome 15:307-314.

Pan X, Eathiraj S, Munson M, Lambright DG. 2006. TBC-domain GAPs for Rab GTPases accelerate GTP hydrolysis by a dual-finger mechanism. Nature 442:303-306.

Pastrana E, Cheng LC, Doetsch F. 2009. Simultaneous prospective purification of adult subventricular zone neural stem cells and their progeny. Proc Natl Acad Sci U S A 106:6387-6392.

Pavlos NJ, Jahn R. 2011. Distinct yet overlapping roles of Rab GTPases on synaptic vesicles. Small GTPases 2:77-81.

Pereira-Leal JB, Hume AN, Seabra MC. 2001. Prenylation of Rab GTPases: molecular mechanisms and involvement in genetic disease. FEBS Lett 498:197-200.

Pereira-Leal JB, Seabra MC. 2001. Evolution of the Rab family of small GTP-binding proteins. J Mol Biol 313:889-901.

Pfeffer S. 2005. A model for Rab GTPase localization. Biochem Soc Trans 33:627-630.

Pfeffer S, Aivazian D. 2004. Targeting Rab GTPases to distinct membrane compartments. Nat Rev Mol Cell Biol 5:886-896.

Pochampalli MR, Bejjani RME, Schroeder JA. 2007. MUC1 is a novel regulator of ErbB1 receptor trafficking. Oncogene 26:1693-1701.

Poteryaev D, Datta S, Ackema K, Zerial M, Spang A. 2010. Identification of the switch in early-to-late endosome transition. Cell 141:497-508.

Raponi E, Agenes F, Delphin C, Assard N, Baudier J, Legraverend C, Deloulme JC. 2007. S100B expression defines a state in which GFAP-expressing cells lose their neural stem cell potential and acquire a more mature developmental stage. Glia 55:165-177.

Recchi C, Seabra MC. 2012. Novel functions for Rab GTPases in multiple aspects of tumour progression. Biochem Soc Trans 40:1398-1403.

Rink J, Ghigo E, Kalaidzidis Y, Zerial M. 2005. Rab conversion as a mechanism of progression from early to late endosomes. Cell 122:735-749.

Rivera-Molina FE, Novick PJ. 2009. A Rab GAP cascade defines the boundary between two Rab GTPases on the secretory pathway. Proceedings of the National Academy of Sciences 106:14408-14413.

Rodriguez-Gabin AG, Cammer M, Almazan G, Charron M, Larocca JN. 2001. Role of rRAB22b, an oligodendrocyte protein, in regulation of transport of vesicles from trans Golgi to endocytic compartments. J Neurosci Res 66:1149-1160.

Rodriguez-Gabin AG, Ortiz E, Demoliner K, Si Q, Almazan G, Larocca JN. 2010. Interaction of Rab31 and OCRL-1 in oligodendrocytes: its role in transport of mannose 6-phosphate receptors. J Neurosci Res 88:589-604.

Rodriguez-Gabin AG, Yin X, Si Q, Larocca JN. 2009. Transport of mannose-6-phosphate receptors from the trans-Golgi network to endosomes requires Rab31. Exp Cell Res 315:2215-2230.

Rodriguez-Gabin A, Almazan G, Larocca J. 2004. Vesicle transport in oligodendrocytes: probable role of Rab40c protein. J Neurosci Res 76:758-770.

Roepstorff K, Grandal MV, Henriksen L, Knudsen SLJ, Lerdrup M, Grøvdal L, Willumsen BM, van Deurs B. 2009. Differential effects of EGFR ligands on endocytic sorting of the receptor. Traffic 10:1115-1127.

Sann SB, Crane MM, Lu H, Jin Y, McCabe BD. 2012. Rabx-5 regulates RAB-5 early endosomal compartments and synaptic vesicles in C. elegans. PloS one 7:e37930.

Sato M, Sato K, Fonarev P, Huang CJ, Liou W, Grant BD. 2005. Caenorhabditis elegans RME-6 is a novel regulator of RAB-5 at the clathrin-coated pit. Nat Cell Biol 7:559-569.

Schardt A, Brinkmann BG, Mitkovski M, Sereda MW, Werner HB, Nave KA. 2009. The SNARE protein SNAP-29 interacts with the GTPase Rab3A: Implications for membrane trafficking in myelinating glia. J Neurosci Res 87:3465-3479.

Schwartz SL, Cao C, Pylypenko O, Rak A, Wandinger-Ness A. 2008. Rab GTPases at a glance. J Cell Sci 121:246-246.

Seabra MC, Coudrier E. 2004. Rab GTPases and myosin motors in organelle motility. Traffic 5:393-399.

Segev N. 2001. Ypt/rab gtpases: regulators of protein trafficking. Sci STKE 2001:RE11.

Segev N. 2011. Coordination of intracellular transport steps by GTPases. Biol 22:33-38.

Sen A, Madhivanan K, Mukherjee D, Aguilar RC. 2012. The epsin protein family: coordinators of endocytosis and signaling. BioMolecular Concepts 3:.

Serão NVL, Delfino KR, Southey BR, Beever JE, Rodriguez-Zas SL. 2011. Cell cycle and aging, morphogenesis, and response to stimuli genes are individualized biomarkers of glioblastoma progression and survival. BMC Medical Genomics 4:49.

Seri B, García-Verdugo JM, McEwen BS, Alvarez-Buylla A. 2001. Astrocytes give rise to new neurons in the adult mammalian hippocampus. J Neurosci 21:7153-7160.

Sigismund S, Argenzio E, Tosoni D, Cavallaro E, Polo S, Di Fiore PP. 2008. Clathrin-mediated internalization is essential for sustained EGFR signaling but dispensable for degradation. Dev Cell 15:209-219.

Simonsen A. 1999. The Rab5 Effector EEA1 Interacts Directly with Syntaxin-6. Journal of Biological Chemistry 274:28857-28860.

Simonsen A, Lippé R, Christoforidis S, Gaullier JM, Brech A, Callaghan J, Toh BH, Murphy C, Zerial M, Stenmark H. 1998. EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. Int Rev Cytol 394:494-498.

Simpson JC, Griffiths G, Wessling-Resnick M, Fransen JAM, Bennett H, Jones AT. 2004. A role for the small GTPase Rab21 in the early endocytic pathway. J Cell Sci 117:6297-6311.

Sivars U, Aivazian D, Pfeffer SR. 2003. Yip3 catalyses the dissociation of endosomal Rab-GDI complexes. Nature 425:856-859.

Soldati T, Shapiro AD, Svejstrup AB, Pfeffer SR. 1994. Membrane targeting of the small GTPase Rab9 is accompanied by nucleotide exchange. Nature 369:76-78.

Song H, Stevens CF, Gage FH. 2002. Astroglia induce neurogenesis from adult neural stem cells. Nature 417:39-44.

Sorkin A, Goh LK. 2009. Endocytosis and intracellular trafficking of ErbBs. Exp Cell Res 315:683-696.

Sorkin A, von Zastrow M. 2009. Endocytosis and signalling: intertwining molecular networks. Nat Rev Mol Cell Biol 10:609-622.

Stenmark H. 2009. Rab GTPases as coordinators of vesicle traffic. Nat Rev Mol Cell Biol 10:513-525.

Strom M. 2002. A family of Rab27-binding proteins. Melanophilin links Rab27a and myosin Va function in melanosome transport. J Biol Chem 277:25423-25430.

Su X, Kong C, Stahl PD. 2007. GAPex-5 mediates ubiquitination, trafficking, and degradation of epidermal growth factor receptor. J Biol Chem 282:21278-21284.

Sun Y, Goderie SK, Temple S. 2005. Asymmetric distribution of EGFR receptor during mitosis generates diverse CNS progenitor cells. Neuron 45:873-886.

Suvorova ES. 2002. The Sec34/Sec35p complex, a Ypt1p effector required for retrograde intra-Golgi trafficking, interacts with Golgi SNAREs and COPI vesicle coat proteins. J Cell Biol 157:631-643.

Tall GG, Barbieri MA, Stahl PD, Horazdovsky BF. 2001. Ras-activated endocytosis is mediated by the Rab5 guanine nucleotide exchange activity of RIN1. Dev Cell 1:73-82.

Tang BL. 2010. Is Rab25 a tumor promoter or suppressor--context dependency on RCP status? Tumour Biol 31:359-361.

Tarafder AK, Wasmeier C, Figueiredo AC, Booth AEG, Orihara A, Ramalho JS, Hume AN, Seabra MC. 2011. Rab27a targeting to melanosomes requires nucleotide exchange but not effector binding. Traffic 12:1056-1066.

Tong J, Sydorskyy Y, St-Germain JR, Taylor P, Tsao MS, Moran MF, Buday L. 2013. Odin (ANKS1A) modulates EGF receptor recycling and stability. PloS one 8:e64817.

Topp JD, Gray NW, Gerard RD, Horazdovsky BF. 2004. Alsin is a Rab5 and Rac1 guanine nucleotide exchange factor. J Biol Chem 279:24612-24623.

Tropepe V, Sibilia M, Ciruna BG, Rossant J, Wagner EF, van der Kooy D. 1999. Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. Dev Biol 208:166-188.

Ullrich O, Horiuchi H, Bucci C, Zerial M. 1994. Membrane association of Rab5 mediated by GDP-dissociation inhibitor and accompanied by GDP/GTP exchange. Nature 368:157-160.

Ullrich O, Stenmark H, Alexandrov K, Huber LA, Kaibuchi K, Sasaki T, Takai Y, Zerial M. 1993. Rab GDP dissociation inhibitor as a general regulator for the membrane association of rab proteins. J Biol Chem 268:18143-18150.

van der Bliek AM. 2005. A sixth sense for Rab5. Nat Cell Biol 7:548-550.

Vanlandingham PA, Ceresa BP. 2009. Rab7 regulates late endocytic trafficking downstream of multivesicular body biogenesis and cargo sequestration. J Biol Chem 284:12110-12124.

Verhoeven K, De Jonghe P, Coen K, Verpoorten N, Auer-Grumbach M, Kwon JM, FitzPatrick D, Schmedding E, De Vriendt E, Jacobs A. 2003. Mutations in the small GTP-ase late endosomal protein RAB7 cause Charcot-Marie-Tooth type 2B neuropathy. Am J Hum Genet 72:722-727.

Wang C, Xin X, Xiang R, Ramos FJ, Liu M, Lee HJ, Chen H, Mao X, Kikani CK, Liu F, Dong LQ. 2009. Yin-Yang regulation of adiponectin signaling by APPL isoforms in muscle cells. J Biol Chem 284:31608-31615.

Wang L, Liang Z, Li G. 2011. Rab22 controls NGF signaling and neurite outgrowth in PC12 cells. Mol Biol Cell 22:3853-3860.

Wang Y, Pennock S, Chen X, Wang Z. 2002. Endosomal signaling of epidermal growth factor receptor stimulates signal transduction pathways leading to cell survival. Mol Cell Biol 22:7279-7290.

Wilde A, Beattie EC, Lem L, Riethof DA, Liu SH, Mobley WC, Soriano P, Brodsky FM. 1999. EGF receptor signaling stimulates SRC kinase phosphorylation of clathrin, influencing clathrin redistribution and EGF uptake. Cell 96:677-687.

Wu YW, Oesterlin LK, Tan KT, Waldmann H, Alexandrov K, Goody RS. 2010. Membrane targeting mechanism of Rab GTPases elucidated by semisynthetic protein probes. Nat Chem Biol 6:534-540.

Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, Sugiyama T, Miyagishi M, Hara K, Tsunoda M. 2003. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. Nature 423:762-769.

Yang X, Zhang Y, Li S, Liu C, Jin Z, Wang Y, Ren F, Chang Z. 2012. Rab21 attenuates EGF-mediated MAPK signaling through enhancing EGFR internalization and degradation. Biochem Biophys Res Commun 421:651-657.

Ying H, Zheng H, Scott K, Wiedemeyer R, Yan H, Lim C, Huang J, Dhakal S, Ivanova E, Xiao Y, Zhang H, Hu J, Stommel JM, Lee MA, Chen AJ, Paik JH, Segatto O, Brennan C, Elferink LA, Wang YA, Chin L, DePinho RA. 2010. Mig-6 controls EGFR trafficking and suppresses gliomagenesis. Proceedings of the National Academy of Sciences 107:6912-6917.

Yoshikawa M, Kajiho H, Sakurai K, Minoda T, Nakagawa S, Kontani K, Katada T. 2008. Tyr-phosphorylation signals translocate RIN3, the small GTPase Rab5-GEF, to early endocytic vesicles. Biochem Biophys Res Commun 372:168-172.

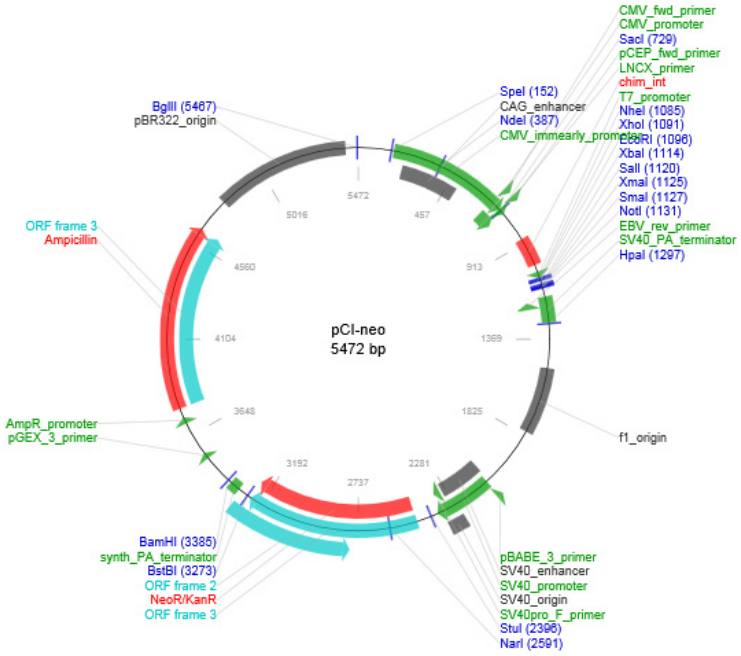
Zhang XM, Ellis S, Sriratana A, Mitchell CA, Rowe T. 2004. Sec15 is an effector for the Rab11 GTPase in mammalian cells. J Biol Chem 279:43027-43034.

Zhu H, Liang Z, Li G. 2009. Rabex-5 is a Rab22 effector and mediates a Rab22-Rab5 signaling cascade in endocytosis. Mol Biol Cell 20:4720-4729.

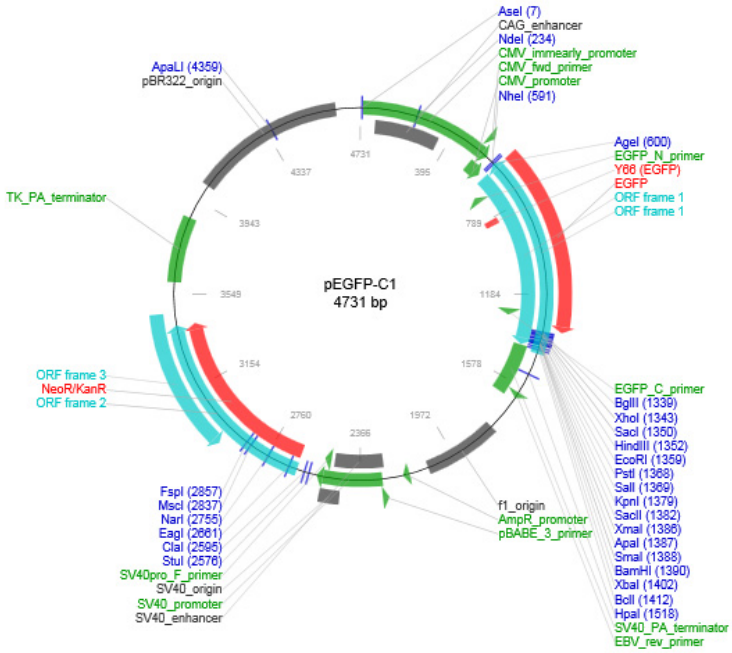
Zoncu R, Perera RM, Balkin DM, Pirruccello M, Toomre D, De Camilli P. 2009. A phosphoinositide switch controls the maturation and signaling properties of APPL endosomes. Cell 136:1110-1121.

Appendix 1 – Plasmid vectors

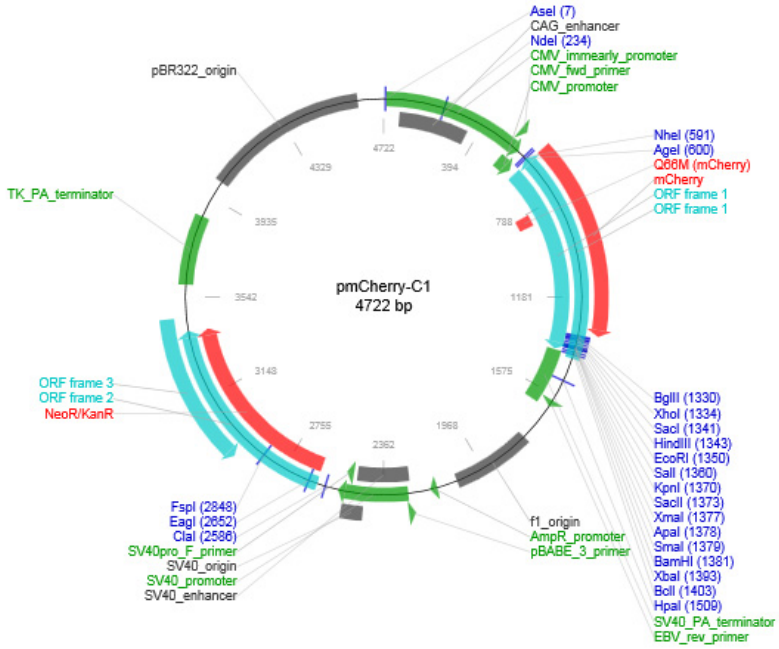
Plasmid maps of vactors used for mammalian gene expression. Images are reproduced from the Addgene (Cambridge, MA) resource, Addgene Vector Database at http://www.addgene.org/vector-database



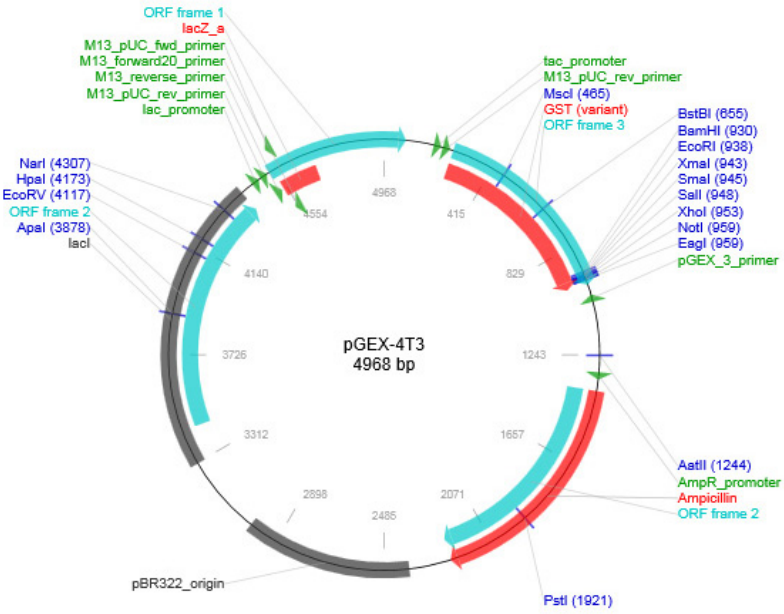
pCI-neo: Expression is driven by the human cytomegalovirus (CMV) immediate-early enhancer/promoter. Transfected cells can be selected with the antiboiotic G418. The expression vector *pDMyc* is modified from *pCI-neo* to include the myc expression tag between the CMV promoter and the multiple cloning site.



pEGFP-C1: Expression is driven by the human cytomegalovirus (CMV) immediate-early enhancer/promoter. Transfected cells can be selected with the antiboiotic G418.



pmCherry-C1: Expression is driven by the human cytomegalovirus (CMV) immediate-early enhancer/promoter. Transfected cells can be selected with the antiboiotic G418.



pGEX-4T3: Bacterial expression is driven by the tac promoter. Amicillin resistance serves as a selection marker.

Appendix 2 – List of Publications

Chua C, Gan B, Tang B. 2011. Involvement of members of the Rab family and related small GTPases in autophagosome formation and maturation. Cell Mol Life Sci. 68:3349-3358.

Chua C, Tang B. 2011. Rabs, SNAREs and α-synuclein--membrane trafficking defects in synucleinopathies. Brain Res Rev. 67:268-281.

Lim Y, **Chua C**, Tang B. 2011. Rabs and other small GTPases in ciliary transport. Biol Cell. 103:209-221.

Chua C, Chan S, Tang B. 2014. Non-cell autonomous or secretory tumour suppression. J Cell Physiol. 229:1346-1352

Chua C, Tang B. 2014. Engagement of small GTPase Rab31 protein and its effector, Early Endosome Antigen 1, is important for the trafficking of ligand-bound Epidermal Growth Factor Receptor from early endosome to late endosome. J Biol Chem. 289:12375-12389

Chua C, Goh E, Tang B. 2014. Rab31 is expressed in Neural Progenitor Cells and plays a role in their differentiation. FEBS Lett. *(In Press)* doi:10.1016/j.febslet.2014.06.060